# The Use of Non–Mammalian Infection Models to Study the Pathogenicity of Members of the Genus *Burkholderia* and *Pseudomonas aeruginosa*

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# I. Summary

*Pseudomonas aeruginosa* and memebers of the *Burkholderia cepacia* complex (Bcc) are important opportunistic pathogens, particularly for immunocompromised individuals and patients suffering from cystic fibrosis. Moreover, these bacteria can cause infections in a diverse range of species, including animals, nematodes, and plants. This allowed the development of various infection models, using the mouse or rat, the nematode *Caenorhabditis elegans*, larvae of the greater wax moth *Galleria mellonella*, *Drosophila melanogaster* or the alfalfa plant as an infection host. In the past few years non-mammalian infection models have become particularly attractive because they are fast, cheap and often allow high throughput screenings. In this thesis I have used and evaluated *C. elegans, G. mellonella* and *D. melanogaster* as infection hosts to investigate the virulence of various *P. aeruginosa* and Bcc wild type and mutant strains to identify virulence factors and to understand their importance in disease.

I could show that some virulence factors are important for pathogenicity in more than one infection model, while other factors were found to be host specific. *N*-Acyl homoserine lactone (AHL)-dependent quorum sensing (QS), a cell-to-cell communication system that is widespread among Gram-negative bacteria, was identified as a highly conserved regulatory mechanism for expression of pathogenic traits both in Bcc strains and in the environmental *P. aeruginosa* isolate PUPa3. For the latter strain it could be shown that the two signaling systems present in this organism operate in parallel, in contrast to the clinical isolate PAO1, in which they are hierarchically arranged. In the case of *B. cenocepacia* it could be shown that siderophore production and intact LPS (lipopolysaccharide) were important for virulence in most animal models. However, I also identified several virulence factors that were required for pathogenesis in only one of the infection hosts.

A major line of my research was dedicated to the identification of virulence factors of *B. cenocepacia* H111. To this end a random mini-Tn*5* insertion library was screened in the *C. elegans* infection model for strains with attenuated pathogenicity. Surprisingly, none of the 22 identified mutants was defective in the biosynthesis of classical virulence factors, such proteolytic enzymes, type III, type IV or type VI secretion systems etc., rather they were found to carry mutations in central metabolic pathways. Specifically, the mutations were mapped to *purA*, *purD*, *purF*, and *purL* encoding enzymes required for purine biosynthesis, *pyrD* required for pyrimidine biosynthesis and *aroK* encoding a key enzyme of the shikimate pathway. These mutants were also attenuated in *D. melanogaster* and *G. mellonella*,

suggesting that these pathways could be valuable targets for the development of novel antimicrobials targeting *Burkholderia*. Another interesting result of the screen was the discovery that 3 of the attenuated mutants had lost chromosome 3 (c3). Subsequent work provided evidence that c3 is not an essential chromosomal element but a large plasmid that encodes virulence, secondary metabolism and other accessory functions in Bcc bacteria.

# II. Zusammenfassung

*Pseudomonas aeruginosa* und Stämme des *Burkholderia* cepacia Komplexes (Bcc) sind gefährliche opportunistische Pathogene für immungeschwächte Personen und Patienten die an cystischer Fibrose leiden. Die Bakterien können zudem auch für Infektionen bei Tieren und Pflanzen verantwortlich sein. Die Eigenschaften diverser Wirte kann genutzt werden, um die Pathogenität in verschiedenen Infektionsmodellen wie z.B. der Maus oder der Ratte, des Nematoden *Caenorhabditis elegans*, der grossen Wachsmotte *Galleria mellonella*, der Fruchtfliege *Drosophila melanogaster*, des Zebrafisches oder der Luzerne zu untersuchen. In den letzten Jahren gewannen wirbellose Wirtstiere in der Forschung immer mehr an Bedeutung, da schnell Ergebnisse generiert werden können; sie sind zudem sehr billig und auch Massenscreenings sind möglich. In dieser Doktorarbeit habe ich *C. elegans*, *G. mellonella* und *D. melanogaster* als Wirtsorganismen verwendet um die Virulenzfaktoren von verschiedenen *P. aeruginosa* und Bcc Wildtypen sowie deren Mutanten zu untersuchen und zu identifizieren um somit die Erkrankung an diesen Bakterien besser zu verstehen.

Ich konnte zeigen, dass einige Virulenzfaktoren für die Pathogenität in mehreren Infektionsmodellen verantwortlich sind, während andere sehr wirtsspezifisch sein können. Das N-Acyl Homoserinlacton (AHL) abhängige Quorum sensing (QS) System, welches der Zell-zu-Zell Kommunikation dient, ist bei Gram-negativen Bakterien weit verbreitet. Für Stämme des Bcc als auch für den Umweltstamm *P aeruginosa* PUPa3 konnte aufgezeigt werden, dass das QS System hoch konserviert und für die Expression von Pathogenitätsfaktoren verantwortlich ist. Sowohl *P. aeruginosa* PUPa3 als auch das klinische Isolat *P. aeruginosa* PAO1 besitzen beide ein zweites QS System. Während die beiden Signalsysteme in PAO1 hierarchisch angeordnet sind, konnte mit Hilfe der Modellorganismen *C. elegans* und *G. mellonella* gezeigt werden, dass diese im PUPa3 Stamm unabhängig voneinander arbeiten. Für *Burkholderia cenocepacia* wurde gezeigt, dass die Siderophorproduktion und intakte Lipopolysaccharide (LPS ) wichtig für die Pathogenität in den meisten Tiermodellen sind. Zudem konnte ich aufzeigen, dass einige Virulenzfaktoren sehr Wirtsspezifisch sind.

Die Hauptaufgabe dieser Studie war die Identifikation von Virulenzfaktoren von *B. cenocepacia*. Zu diesem Zweck wurden die Mutanten einer mini-Tn*5* Mutantenbank mit Hilfe des Nematoden *C. elegans* auf deren Pathogenität untersucht und die attenuierten Stämme wurden weiter untersucht. Erstaunlicherweise befand sich keine der Mutationen der 22 gefundenen Stämme in Genen, welche für die Biosynthese klassischer Virulenzfaktoren wie

proteolytischer Enzyme, TypIII, TypIV oder TypVI Sekretionssysteme usw. verantwortlich sind. Dafür trugen viele der gefundenen Stämme Mutationen in zentralen metabolischen Stoffwechselwegen. Insbesondere wurden Mutationen im Purin Syntheseweg (*purA*, *purD*, *purF*, und *purL*), *pyrD*, welcher für die Pyrimidinbiosynthese erforderlich ist und *aroK*, welches eines Schlüsselenzymes im Shikimatsyntheseweges kodiert, gefunden. All diese aufgelisteten Mutanten waren auch in *D. melanogaster* und *G. mellonella* attenuiert. Somit könnten diese Stoffwechselwege von besonderem Interesse für die Entwicklung neuartiger antimikrobieller Medikamente und zur Bekämpfung von *Burkholderien* sein. Eine weitere interessante Entdeckung des Screens war, dass drei der attenuierten Mutanten das ganze Chromosom 3 (C3) verloren hatten. Durch weitere Forschung an diesen Stämmen konnte gezeigt werden, dass C3 durch seine Eigenschaften eher als riesiges Plasmid zu bezeichnen ist, welches unter anderem Virulenzfaktoren, sekundär Metaboliten und andere Zusatzfunktionen in Bcc Bakterien kodiert.

# III. Abbreviations

| | |
|---|---|
| AHL | *N*-acyl homoserine lactone |
| AMPs | antimicrobial peptides |
| Bcc | *Burkholderia cepacia* complex |
| bp | base pair |
| CF | Cystic fibrosis |
| CFU | colony-forming units |
| °C | degree Celsius |
| C | cytosine |
| EPS | extracellular polymeric substances |
| *et al.* | et alii |
| G | guanine |
| h | hours |
| IL-1 | interleukin-1 receptor |
| IMP | inosine monophosphate |
| $LD_{50}$ | Lethal Dose, 50% |
| LPS | lipopolysaccharide |
| OD | optical density |
| % | percent |
| PVC | Polyvinylchloride |
| Mb | mega base |
| µg | microgram |
| mg | milligram |
| min | minute |
| µl | microliter |
| ml | millilitre |
| mm | millimetre |
| mM | millimolar |
| MQ | Milli-Q water |
| NGM | nematode growth medium |
| QS | quorum sensing |
| Rpm | revolutions per minute |
| SAP | shrimp alkaline phosphatase |
| TLC | Toll-like receptor |
| VF | virulence factor |
| w/v | weight per volume |

# 1 Introduction

## 1.1 *Burkholderia*

*Burkholderia* are Gram-negative, non-spore forming, rod-shaped bacteria that belong to the β-subgroup of the proteobacteria. They were first isolated and described as *Pseudomonas cepacia* in the 1950ies by W. H. Burkholder as a bacterium that causes "sour skin", a disease of onion bulbs (Burkholder, 1950). With the improvement of molecular methods for bacterial identification seven species from the *Pseudomonas* genus were transferred to a new genus that was named *Burkholderia* (Yabuuchi *et al.*, 1992). Currently, the genus comprises more than 70 species which have been isolated from various ecological niches, including soil and freshwater. Many strains have been shown to be able to colonize plant roots, produce antibiotics and some strains are capable of fixing atmospheric nitrogen (Cruz *et al.*, 2001; Estrada-De los Santos *et al.*, 2001) and/or form root nodules (Barrett & Parker, 2005; Barrett & Parker, 2006; Chen *et al.*, 2005). Many of these strains exhibit the ability to promote plant growth (Parke & Gurian-Sherman, 2001). The enormous variability of this genus is reflected in its large genome with size ranging from 4.7 up to 9 Mb (Lessie *et al.*, 1996). *Burkholderia* strains have been reported to use polychlorinated biphenyles as carbon and energy sources (Nogales *et al.*, 1999), they are able to degrade herbicides (Kilbane *et al.*, 1982), pesticides (Holmes *et al.*, 1998) and trichloroethylene (Folsom *et al.*, 1990), can grow on PVC (Drabick *et al.*, 1996), colonize stainless steel (Vaisanen *et al.*, 1998), grow in colour solutions (Gravel *et al.*, 2002; Morel *et al.*, 2003), disinfectants (Garcia-Erce *et al.*, 2002; Oie & Kamiya, 1996), raw milk (Morel *et al.*, 2003) and gelatine (De Clerck & De Vos, 2002). Due to these characteristics some strains have attracted attention for the bioremediation of contaminated soils, as bio-control agents and as plant growth promoting bacteria (Coenye & Vandamme, 2003). On the other hand, some *Burkholderia* species are known as plant pathogens. *B. mallei* and *B. pseudomallei* are primary pathogens of humans and animals (Cheng & Currie, 2005). Strains belonging to the *Burkholderia cepacia* complex (Bcc; see below) can act as opportunistic pathogens for immunocompromised individuals and patients suffering from cystic fibrosis or chronic granulomatous disease (CGD).

The Bcc consists of 17 species which are phenotypically very similar but genetically versatile. These 17 species were defined by polyphasic taxonomy approaches, including fatty acid analysis, whole-cell protein profile analysis, 16S rRNA and *recA* gene sequencing and DNA-DNA hybridization (Vandamme & Dawyndt, 2011). These stains share a high degree of

rRNA (98- 100%), *recA* sequence (94- 95%) similarity but only a moderate level of DNA-DNA similarity of 30- 50%. The currently described Bcc species are; *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia*, and *B. ubonensis*, *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica*, *B. lata* and *B. contaminans* (Coenye *et al.*, 2001; Coenye & Vandamme, 2003; Vandamme *et al.*, 1997; Vanlaere *et al.*, 2008; Vanlaere *et al.*, 2009; Vermis *et al.*, 2004).

## 1.2 Bcc infections and cystic fibrosis

Cystic fibrosis (CF) is a genetically, autosomal recessive inherited metabolic disease. It is one of the most common life-shortening genetic defects in the population of European origin, affecting 1 in 2500 birth (Bye *et al.*, 1994). The symptoms of this metabolic defect are cause by mutations in a gene on chromosome 7, the **c**ystic **f**ibrosis **t**rans **m**embrane **c**onductance **r**egulator (CFTR), which encodes a transepitelial chloride channel (Drumm *et al.*, 1990). The disfunction of this ion channel leads to an altered liquid household, the lung gets dehydrated, leading to the production of a highly viscous mucus. This mucus and the associated bacteria cannot be easily removed by the cilia (Pilewski & Frizzell, 1999) causing chronic cough, chronic hepatic disease, lung insufficiency and intestinal obstructions (Rosenstein & Zeitlin, 1998). In the CF lung the conditions for bacterial colonization are therefore ideal. In the early childhood CF patients are often infected with *Staphylococcus aureus* followed by *Haemophilus influenza* and *Pseudomonas aeruginosa*. 80% of the lungs of adult CF patients are colonized by *P. aeruginosa* (Govan & Deretic, 1996). In the 80ies the first cases of infection with *B. cepacia* were observed in patients suffering from CF. *Burkholderia* co-infections with *P. aeruginosa* can become chronically. Approximately 20% of the patients infected with Bcc strains succumb to a fast and fatal necrotizing pneumonia the so called cepacia syndrome (Isles *et al.*, 1984). CF patients which are colonized with Bcc strains have a reduced like expectancy after lung transplantation (Aris *et al.*, 2001). Another problem is the inherent multi antibiotic resistance of Bcc strains and the transmission of certain epidemic strains from patient to patient. For this reason, patients have to be separated to avoid close contact from each other (Duff, 2002; Lipuma *et al.*, 1990).

## 1.3 Pathogenicity models for memebers of the genera *Burkholderia* and *Pseudomonas*.

To test the pathogenicity of Bcc strains as well as of *P.* aeruginosa, many infection hosts have been used. The first plant model used to investigate the pathogenicity of *Burkholderia* was the onion *Allium cepa.* To this end onion slices were inoculated with the strains of interest and tissue maceration was recorded (Gonzalez & Vidaver, 1979). As another plant infection model the lucerne alfalfa has been established. The seeds are germinated under sterile conditions and wounded leaves are infected with the strain of interest. Following infection the plants are incubated at 37°C and the level of pathogenicity is determined (Bernier *et al.*, 2003; Silo-Suh *et al.*, 2002). For *Pseudomonas aeruginosa* additional plant hosts have been used to study virulence, including *Arabidopsis thaliana* and lettuce (*Lettuca sativa*) plants (Rahme *et al.*, 1997). With both models the plant leaves are infected with the pathogen and the damage of the infection as well as the bacterial cell numbers can be determined (Prithiviraj *et al.*, 2005; Rahme *et al.*, 1997; Starkey & Rahme, 2009). Rhizosphere virulence factors can be studied with the barley seed pathogenicity assay, in which the seeds are germinated together with the bacteria and the number of germinated seeds can be counted after three days (Attila *et al.*, 2008). The benefits by using plants as infection models are the low costs and the easy and fast growing of the plants, which makes large screens of many bacterial strains in a short time possible. Moreover, the genome of *Arabidopsis thaliana* has been sequenced and many different mutants are available and can be used to also study host responses (Starkey & Rahme, 2009).

The Sprangue-Dawley rat serves as an animal infection host. To establish an infection the bacteria are embedded in agar and inserted into the lung *via* a cut in the trachea. Seven to 21 days later the pathogenicity of the strains is determined by quantifying the inflammation of the lungs (Cash *et al.*, 1979). A so-called CF mouse ($Cftr^{tm1Unc}$ or $Cftr^{tm1Hgu}$ mouse) has also been described, which usually is infected by exposure of the animals to aerosolized cultures of interest. Alternatively, the mice are anesthetized and the bacteria are instilled drop wise intranasally and are then aspirated into the lungs (Davidson *et al.*, 1995; Sajjan *et al.*, 2001). Another mouse mutant used in infection studies is the CDG mouse, which has a null allele of the gene involved in X-linked **c**hronic **g**ranulomatous **d**isease. In this case, the animals are anesthetized and infected with the pathogens by intratracheal inoculation *via* mid-line cervical incision. The infection normally leads to the development of neutrophil-dominated lung abscesses (Sousa *et al.*, 2007). Furthermore, to study the clearance or persistence of Bcc, a leukopenic mouse model has been established. For this BALC/c mice have been used as

infection hosts. To achieve a mild leukopenia, the mice were treated by admistering low dosages of cyclophosphamide before infection. Here, the mice are infected *via* intranasal instillation. At given time points the mice are killed by cervical dislocation and the lungs are weighted, homogenized and plated onto TSA plates for determination of bacterial counts (Chu *et al.*, 2002). The virulence of the different strains used in the mice models can be determined by histopathological inspection or by determination of the bacterial load.

A vertebrate model organism used as infection host is the zebrafish. The bacteria are injected into the embryos of the zebrafish *via* microinjection and are kept in 24-well plates. The mortality is determined every 24h (Vergunst *et al.*, 2010). One of the first non-mammalian infection host, which was used for investigations of the virulence of *P. aeruginosa* and *B. cenocepacia* was the nematode *C. elegans* (Kothe *et al.*, 2003; Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999b). A closely related nematode, *Panagrellus*, has also been used as infection host for *Burkholderia multivorans (Laws et al.,* 2005*)*. The advantage of *P. redivivus* is that this nematode can be incubated at 37°C, while *C. elegans* only tolerates temperatures between 13°C and 25°C for normal development (Hedgecock & Russell, 1975). However, the fact that this nematode is viviparous makes it impossible to synchronize larval stages and thus it is difficult to work with this nematode. Furthermore, the larvae of the greater wax moth *Galleria mellonella* has been established as an infection model for *P. aeruginosa* (Jander *et al.*, 2000) and Bcc strains (Seed & Dennis, 2008; Uehlinger *et al.*, 2009). The advantage of this model is that a known amount of bacteria is injected. Another model organism is the fruit fly *Drosophila melanogaster* for which the possibility of a feeding and pricking assay exists (Apidianakis & Rahme, 2009; Castonguay-Vanier *et al.*, 2010).

As *in vitro* infection models for the identification of virulence factors, the evaluation of pathogenicity mechanisms and the characterization of host responses different macrophage cell lines, epithelial cell line A549, type II pneumocytes, neutrophils and even lung explants have been used (Leitao *et al.*, 2010).

### 1.3.1 *Caenorhabditis elegans*

Because of the short generation time, the compact genome, the stereotypical development, the easy breading, the small size and its easy blueprint (~ 1000 somatic cells at the adult animal), *C. elegans* is one of the most important model organisms in genetics, cell biology, molecular biology, neurobiology, developmental biology. In infection biology *C. elegans* has been used since 1999 to analyse different virulence factors of various bacteria (Tan *et al.*, 1999b). The nematode *C. elegans* is around 1 millimetre long and lives in the soil of temperate zone where it feeds on microorganisms. The optimal temperature for growth is at around 20°C. *C. elegans*

has got two sexes, males (X0) and self-fertilizing hermaphrodites. The males accrue to spontaneous nondisjunction due to non-separation of the chromosomes during the meiosis with a rate of 0.1%. Hermaphrodites produce oocytes and sperms which gives them the possibility of self-fertilizing, which leads to genetically homogenous populations. It has a generation time of 3 to 4 days under optimal conditions and hermaphroditic nematodes can produce between 250 to 300 eggs during a generation time (Ewbank, 2002). The male worms can produce by mating around 1200-1400 off springs (Hodgkin & Doniach, 1997). The normal lifespan is around three weeks and the worms undergo four different larval stadiums (from the egg to an adult worm) which differ only in the size. If the conditions are not optimal, for example a shortage of food, they can undergo a transition from the second to the third stage where they need no food and where they are also resistant against dehydration. In this case they can survive up to three months and when the conditions get better they develop normally (Cassada & Russell, 1975).

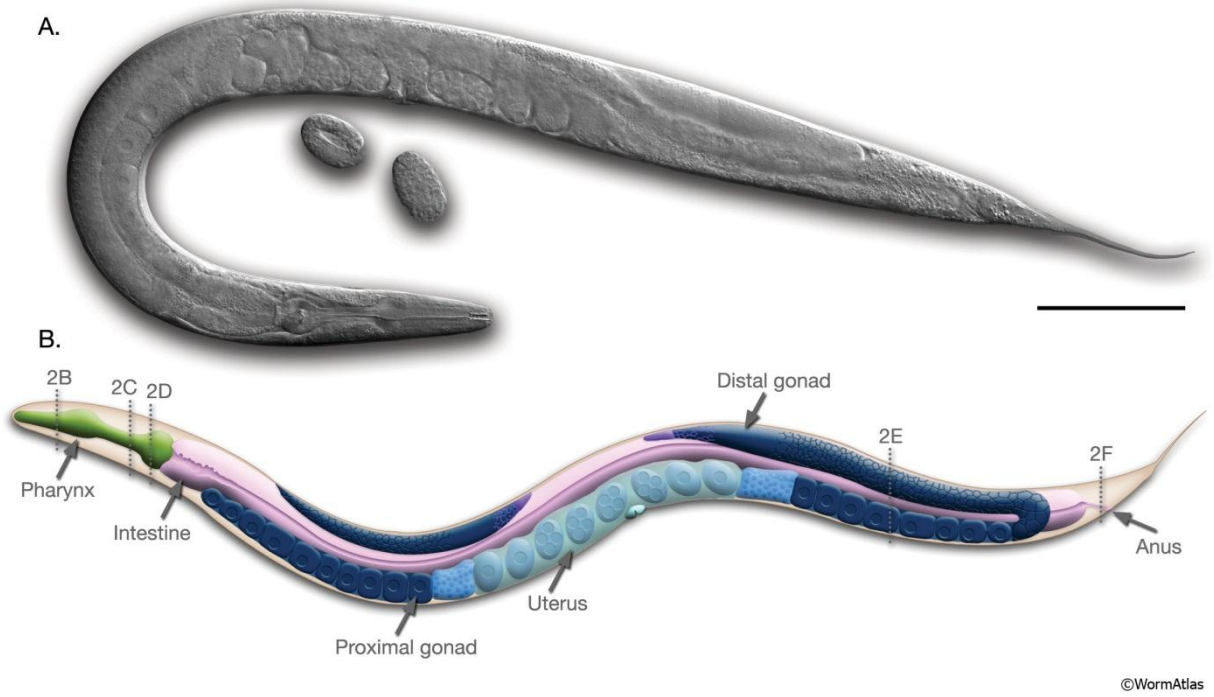


**Figure 1:** On picture **A** differential interference picture of an adult hermaphrodite is shown with a scale bar of 0.1 mm. **B** shows a schematic scheme with the most important anatomical structures of *C. elegans* (www.wormatlas.org).

Due to the fact that it is a very cheap model with no ethical problems (no permission is needed), the genome of the nematode has been determined, the worm has got an innate immune system and high throughput screenings are possible, *C. elegans* has become an important model organism to study the pathogenicity of different human pathogens. The availability of the *C. elegans* genome sequence opens many possibilities to study host-pathogen interactions also from the host side, particularly as various mutants can be accessed through public strain collections. This allows studying the pathways and immune responses important for defence against the invasive pathogen. The first report on *C. elegans* as an infection host was investigating the virulence of clinical isolate *P. aeruginosa* PA14, which was used as food source instead of the non-pathogenic food strain *E. coli* OP50 (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999b). Subsequent work showed that *C. elegans* is also suitable for investigation the virulence of Bcc strains (Koethe *et al.*, 2003). The most limiting factor for the use of this model is the temperature, which cannot be above 25°C (Klass, 1977). Several human pathogens, however, often induce the production of virulence factors specifically at 37°C (Konkel & Tilly, 2000). Moreover, the assessment of virulence in a dose dependent manner is not possible and food avoidance has been described (Zhang *et al.*, 2005). The nematode *C. elegans* is a good and fast model organism to compare the pathogenicity of different strains or mutants by the killing assay. Also the colonization of the gut by the bacteria can be easily evaluated. In this study *C. elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis, USA), has been used.

### 1.3.2 *Galleria mellonella*

The greater wax moth larva is a pest organism in beehives, because they feed on honeycombs, pollen and partially eggs and bee larvae inside bee nests. Besides they can also transmit diseases to the bee colony. The larvae grow up to three centimetres in length and gaining a weight of 300 to 500 mg. The white larvae turn *via* a metamorphosis into a grey moth (Kavanagh & Reeves, 2004). Because they are used often for fishing it is easy to get them from fishing shops.

*G. mellonella* larvae are often used as a model organism to study bacterial pathogenicity because of the similarities of the innate immune systems of insects and mammals (Cotter *et al.*, 2000). The insects have a haemocoel which contains the haemolymphe. The haemolynphe itself contains six different types of haemocytes which have similar functions to phagocytes in mammals and humans. The haemolymphe has a similar function as the blood in mammals, including the transport of nutrients, waste products and signal molecules (Kavanagh &

Reeves, 2004), but it plays no role in respiration. In addition, it contains also antimicrobial peptides and enzymes, which protect the organism against invading microorganisms (Morton *et al.*, 1987; Vilmos & Kurucz, 1998). A very important defence mechanism of the haemolymphe is the melanisation, where melanin gets deposed around the invaded microorganism with the result that the larvae turn black (Kavanagh & Reeves, 2004).

Because of the similarities between the immune systems it has been suggested that *G. mellonella* can be used instead of mice, which could reduce cost and time. Many virulence factors in *P.* aeruginosa were shown to be required for virulence in both *G. mellonella* and mice (Jander *et al.*, 2000). *G. mellonella* has also been used as a model organism for investigating the pathogenicity of *Proteus mirabilis* (Morton *et al.*, 1987), *Candida albicans* (Brennan *et al.*, 2002), *Bacillus cereus* (Kavanagh & Reeves, 2004; Salamitou *et al.*, 2000), *Escherichia coli* (Kavanagh & Reeves, 2004), *Bacillus thuringiensis* (Salamitou *et al.*, 2000), *Cryptococcus neoformans* (Mylonakis *et al.*, 2005), *Francisella tularensis* (Aperis *et al.*, 2007) and for members of the Bcc (Seed & Dennis, 2008; Uehlinger *et al.*, 2009). Beside the low costs and a relative fast screening the big advantages of using *G. mellonella* as a model organism are the ability to conduct the experiment at 37°C and that a known amount of bacteria is injected. Hence, with this infection model it is possible to determine the $LD_{50}$. Furthermore,the multiplication of the bacteria inside the host can be easily determined by plating samples of the hemolymph. Changes in the innate immune response after infection can be assessed by measuring the changes in the haemocyte density (Gagen & Ratcliffe, 1976; Matha & Mracek, 1984) or the expression level of antimicrobial proteins (Vilmos & Kurucz, 1998). A disadvantage by using this model is that its genome has not yet been sequenced.

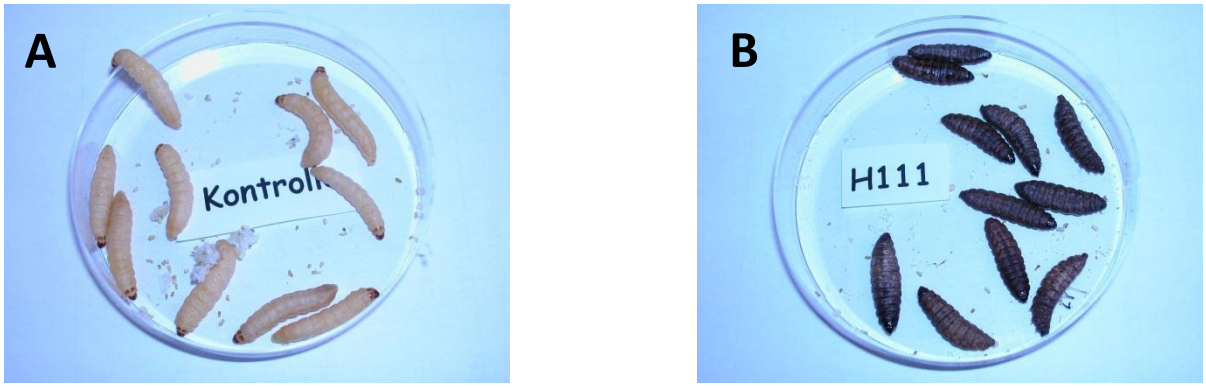


**Figure 2:** *G. mellonella* pathogenicity assay 48 h after infection. **A:** control after infection with 10 µl of $MgSO_4$ buffer. **B:** dead larvae after infection with approximately 350'000 to 400'000 *B. cenocepacia* H111 cells.

### 1.3.3 *Drosophila melanogaster*

The fruit fly *D. melanogaster* is like *C. elegans* relative small, it has a short generation time, it is very cheap, the genome is fully sequenced and many mutants are available. Like with the sequenced nematode *C. elegans* many fly mutants are available, which makes it possible to study the response of the host to the invading pathogen. This model also allows the determination of the bacterial load inside the insect. The flies have a similar innate immune response with similar genes and pathways to those in mammals (Hoffmann, 2003). Examples are the Toll-like receptor (TLC), the interleukin-1 receptors (IL-1) and the tumor necrosis factor pathway Imd (Sekiya *et al.*, 2008). One important factor of the innate immune system is the specialized hemocytes which are similar to red blood cells. They are involved in in phagocytosis and encapsulation of invading microbes (Rizki & Rizki, 1984). The humoral response of the innate immune system includes the production of antimicrobial peptides (AMPs), which are secreted into the haemolymph (Lemaitre, 2004). Approximately 20 AMPs have been discovered so far. These peptides were classified into seven groups: drosomycins and metchnikowins (acting against fungi), defensin (against Gram-positive bacteria) attacin, cecropin diptericin, and drosocin, (against Gram-negative bacteria) (Lemaitre & Hoffmann, 2007).

**Figure 3:** *D. melanogaster* canton S wild type fly during a pricking experiment.

## 1.4 The use of multiple hosts to study bacterial virulence

There are striking parallels in the innate (also referred to as non-adaptive) immunity of plants, invertebrates and mammalians (Kopp & Medzhitov, 1999). Some virulence mechanisms of pathogens and the host defences against them appear to have ancient evolutionary origins (Mahajan-Miklos *et al.*, 2000). For example Rhame *et al.* (1995) has shown that *P. aeruginosa*is capable of infecting plants, nematodes and insects by using the same set of universal virulence factors (Jander *et al.*, 2000; Mahajan-Miklos *et al.*, 1999; Tan *et al.*,

1999a; Tan *et al.*, 1999b; Yorgey *et al.*, 2001) The defense mechanisms of insects includesbehavioral responses, physical barriers and the innate immune system (Schulenburg *et al.*, 2004).

The cuticle of insects is the first line of defence against pathogens. It has similar functions as the mammalian skin and helps to prevent the entry of pathogens into the haemocoel (Clarkson *et al.*, 1998). If the cuticle gets injured, the humoral immune response is activated. The haemocoel of insects contains the haemolymph, which has a similar function as the blood in mammals, including the transport of waste products, nutrients,signal molecules (Matha & Mracek, 1984) and anti-microbial peptides, which are able to immobilize and kill invading microorganisms (Morton *et al.*, 1987; Vilmos & Kurucz, 1998). This makes the haemolymph to the most important element of the immune response against invading microorganisms. The haemocytes of the haemolymph have a similar function as the phagocytes of mammals. Different types of haemocytes like prohaemocytes, plasmatocytes, grandulocytes, coagulocytes (also named hyaline hemocytes), spherulocytes, oenocytoids adipohemocytes, lamellocytes and crystal cells have been described (Brehelin & Zachary, 1986; Price & Ratcliff.Na, 1974). They participate in phagocytosis, form nodules, are involved in the encapsulation and in the clotting process (Ratcliffe, 1993; Tojo *et al.*, 2000), production of antimicrobial peptides (Samakovlis *et al.*, 1990) and in the melanisation process (Nappi & Vass, 1998).

**The cellular immune response** is not fully understood in insects, but receptors of the surface of granulocytes and plasmatocytes bear similar receptors as mammalian phagocytes (Vilmos & Kurucz, 1998). It has been shown, that a 47 kDa protein of *G. mellonella* share a strong homology with calreticulin of humans and *Drosophila*. Calreticulin is localised of the surface of neutrophils. If an invading organism or compound binds to calreticulin, a signal is transduced *via* the G-protein and this leads to the production of superoxide anions (Cho *et al.*, 1999). Furthermore, the humoral recognition receptors can bind to invading material and stimulate the aggregation to plasmocytes (Lavine & Strand, 2001). The **humoral immune response** of insects consists of melanisation, haemolymph clotting, wound healing, the synthesis of anti-microbial peptides and heat shock proteins (Salzet, 2001).

The fact that the insect immune response is not so complex but still very similar as those mammals made insect models extremely popular for studies of host-pathogen interactions(Levy, 2001; Salzet, 2001; Vilmos & Kurucz, 1998) . It allows comparing the effect of specific pathogens in its host in a simpler way (Kavanagh & Reeves, 2004). Such

models also help to reduce the costs, time and the number of mammals used for infection studies. However, due to the fact that the adaptive immune system is absent in insects they will never replace the use of mammals. Antibody response and immunization studies are just two examples which are impossible to investigate in insects without an adaptive immune response.

## 1.5 *P. aeruginosa*

The genus of *Pseudomonas* was first described by Walter Migula in 1894 (Migula, 1894). *P. aeruginosa* has first been cultivated in pure culture by Gessard in 1882 from cutaneous wounds, which showed a blue-green colour (Gessard, 1984). This gave the bacterium the name *aeruginosa*, which is the Latin word for verdigris or copper rust which has a similar colour as the blue-green pigment which the bacterium produced in laboratory cultures. It is a Gram-negative non-spore forming, rod shaped aerobic bacillus which belongs to the γ-subgroup of the proteobacteria (Berra *et al.*, 2010; Hamana & Matsuzaki, 1992) and it can be found ubiquitously distributed in the environment, including soil and water (Schmidt *et al.*, 1996), or as pathogens of plants, animals and humans. *P. aeruginosa* grows at a temperature of 42°C (Haynes, 1951). *P. aeruginosa* PAO1 (***P****seudomonas* ***a****eruginosa* **O**ne) has a G + C content of 67% and a genome size of 6.3 Mb (Stover *et al.*, 2000), which is an average size compared with other *P. aeruginosa* strains (5.2 to 7 Mb) (Tümmler, 2006). This large genome size reflects its ability to adapt to different ecological niches. *P. aeruginosa* can metabolize and grow on a wide range of organic compounds (Green *et al.*, 1974; Williams & Worsey, 1976). *P. aeruginosa* has been isolated from diverse sources including soap, sinks, mops, respiratory therapy equipment, antiseptics, medicines, urinary catheters, home humidifiers, contact lens solution, soil, rhizosphere, vegetables, swimming pools and even from distilled water (Bodey *et al.*, 1983; Doring *et al.*, 1996; Favero *et al.*, 1971; Harris *et al.*, 1984; Lanini *et al.*, 2011; Pitt, 1998; Pollack, 1990). Another problem with this opportunistic pathogen is its antibiotic resistance against many antibiotics (Li *et al.*, 1994a; Li *et al.*, 1994b), which makes treatment of infections difficult. Patient-to-patient transmissions in hospitals *via* contaminated medical devises have been reported (DiazGranados *et al.*, 2009; Iversen *et al.*, 2007; Shimono *et al.*, 2008). *P. aeruginosa* is considered one of the most important and significant opportunistic human pathogens and a source of bacteraemia in burn wound victims, immunocompromised and immunodeficient patients (Bodey *et al.*, 1983; Hakki *et al.*, 2007). For patients suffering from cystic fibrosis (CF) is it the predominant cause for morbidity and mortality.

## 1.6 Quorum sensing: A cell-to-cell communication system

The expression quorum was coined in the Roman Empire. It was the minimum number of members in the senate which were responsible for a valid vote. In microbiology quorum sensing (QS) denotes a cell to cell communication mechanism that depends on the production of small signal molecules (Fuqua *et al.*, 1994). With this system bacteria can coordinate their activities similar to multicellular organisms. The ability to sense the density of the population to a coordinate expression of certain phenotypic traits is widely distributed in the bacterial kingdom (Fuqua *et al.*, 1994). The best investigated signal molecules are small peptides, which are used mostly by Gram-positive bacteria, and the *N*-acyl homoserine lactones (AHLs), which are used by many Gram-negative bacteria. AHLs differ in the length of the acyl chain and their modifications at position C3 (Waters & Bassler, 2005; Whitehead *et al.*, 2001). At low cell density the AHL synthase produces a basal level of AHLs. The concentration of the signal molecules increases together with the bacterial cell density until they reach a threshold concentration which triggers expression or inhibition of target genes. QS-regulated phenotypes include swarming, swimming, biofilm formation, antibiotic biosynthesis, virulence and bioluminescence (Venturi, 2006; Williams *et al.*, 2000).

QS in bacteria was first described for the marine bacterium *Vibrio fischeri*, in which it regulates bioluminescence (Ruby, 1996). The basic mechanism of QS depends on two proteins which belong to the LuxI-LuxR family (Eberl, 2006; Fuqua *et al.*, 2001; Whitehead *et al.*, 2001). The LuxI protein is responsible for the production of AHLs, which are freely diffusible. By reaching a critical AHL concentration, the molecule interacts with the LuxR-type protein. The LuxR/AHL complex binds to specific DNA sequences so-called *lux*-boxes to activate gene expression. In 1999 Lewanza and co-workers identified two genes in *B. cenocepacia* K56-2 named *cepI* and *cepR* which are homologs of *luxI* and *luxR* (Lewenza *et al.*, 1999). The authors showed that CepI directs the synthesis of two AHL molecules, *N*-octanoyl-homoserine lactone (C8-HSL) and as a minor product the *N*-hexanoyl-homoserine lactone (C6-HSL). The transcription of *cepI* is autoregulated and the AHL molecules are thought to be exported by Resistance-Nodulation-Cell Devision (RND) efflux pumps (Buroni *et al.*, 2009). By reaching a critical threshold, the AHL signal molecules binds to its receptor CepR. This complex is able to activate or repress the transcription of different target genes. The production of chitinases, extracellular proteases, the synthesis of the nematocidal protein AidA, biofilm formation, swarming motility, the biosynthesis of the siderophore pyochelin and of virulence factors are examples for positively QS-regulated functions (Huber *et al.*, 2001; Huber *et al.*, 2002; Huber *et al.*, 2004; Koethe *et al.*, 2003; Malott *et al.*, 2009).

Systematic screening of different Bcc strains showed that all species of the Bcc contain CepI/CepR QS systems that utilize C8-HSL (Gotschlich *et al.*, 2001; Lutter *et al.*, 2001). Some strains contain a second QS system. Strains from the epidemic ET12 linage like *B. cenocepacia* K56-2 encode a 31.7 kb-large pathogenicity island on chromosome 2, which carries an additional QS system, the so-called CciIR system, which mainly utilizes C-6HSL and C8-HSL (Baldwin *et al.*, 2004; Malott *et al.*, 2005). Another member of the Bcc that bears a second system "*bviI*/*bviR*" is *B. vietnamiensis*, which produces *N*-decanoyl homoserine lactone (C10-HLS). However, it is unknown whether these two QS systems interact with each other or not (Conway & Greenberg, 2002; Lutter *et al.*, 2001; Malott & Sokol, 2007).

Another example of an organism harbouring two different QS systems is *P. aeruginosa*: the LasI/R and RhlI/R systems, which are involved in the control of virulence (Fuqua *et al.*, 2001; Lazdunski *et al.*, 2004; Smith & Iglewski, 2003; von Bodman *et al.*, 2003; Whitehead *et al.*, 2001). The LasI is responsible for the production of *N*-(3-oxo-dodecanoyl)-$_L$-homoserine lactone (3-oxo-$C_{12}$-AHL) and this molecule interacts with the LasR receptor. The second system consist of RhlI which produce *N*-(butanoyl)-$_L$-homoserine lactone ($C_4$-AHL) and the RhlR receptor. The binding of these two AHLs to their cognate receptors results in the expression of target genes, including many virulence factors such as alkaline protease, elastase, rhamnolipides, exotoxin A, phycocyanin, superoxide dismutase, lectins and biofilm formation (Smith & Iglewski, 2003). A number of studies have demonstrated, that QS mutants are attenuated in virulence in various infection hosts such as *Arabidopsis thaliana, Dictoystelium discoideum* and *C. elegans* (Juhas *et al.*, 2005; Smith & Iglewski, 2003; Uehlinger *et al.*, 2009).

## 1.7 Goals of this work

The aim of this study was to evaluate the advantages and disadvantages of non-mammalian infection models for investigations on the pathogenicity of *P. aeruginosa* and Bcc strains. As infection hosts *C. elegans, G. mellonella* and *D. melanogaster* were used. In the case of *P. aeruginosa* I investigated the role of the two QS-systems in pathogenesis in the environmental isolate PUPa3 as well as the virulence of *P. aeruginosa* longitudinal isolates from cystic fibrosis patients in *D. melanogaster, C. elegans* and *G. mellonella*. The *D. melanogaster* pricking model should also be employed to screen around 300 strains from a *P. aeruginosa* PAO1 Tn*5* transposon insertion library for attenuated mutants.

Another line of research was the screen of 5500 mutants of a random mini-Tn*5* insertion library of *B. cenocepacia* H111 for strains with reduced pathogenicity toidentify genes encoding novel virulence factors. Three mutants were found to be strongly attenuated in *G. mellonella* and *C. elegans*. A more detailed analysis revealed that these mutants had lost chromosome three (pC3). Another member of the Eberl group, Kirsty Agnoli, constructed pC3 cured mutants of members of the Bcc, and I have collaborated with here to analyse the pathogenicity of these strains in *G. mellonella* and *C.* elegans. In the same screening 22 mutants showed attenuation in the *C. elegans* model. These mutants should be further investigated and also tested in *G. mellonella* and *D. melanogaster* to identify general and host-specific virulence factors. Furthermore inhibitors of identified virulence factors should be tested to identify lead compounds for the development of novel antimicrobial agents.

# 2 Material and Methods

## 2.1 Table 1: Strains plasmids and animals used in this study.

| Strain/plasmid/animals | Characteristics | Reference/source |
|---|---|---|
| ***E. coli*** | | |
| OP 50 | Food source strain for *C. elegans*, uracil auxotrophic | (Brenner, 1974) |
| HB101 | *recA thi pro leu hsd*R$^-$M$^+$ *Sm*$^r$ | (Boyer & Roulland.D, 1969) |
| DH5α | F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAl deoR(U169)* | (Hanahan, 1983), Invitrogen |
| XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F*'proAB lacIq* ZΔM15 Tn*10* (*Tet*$^r$)] | Stratagene |
| MT102 | *araD139( ara-leu) 7679 Δlac thi hsdR* | Laboratory collection |
| ***Pseudomonas putida* F117 (pAS-C8)** | Bioluminescent AHL-biosensor, F117 with pBBR1MCS-5 carrying P*cepI*::*gfp*(ASV) P*lac*::*cepR;* Gmr | (Steidle *et al.*, 2001) |
| ***Burkholderia cenocepacia*** | | |
| H111 | CF-isolate, Germany | (Romling *et al.*, 1994) |
| H111-I | H111*cepI*::Km mutant, Km$^R$ | (Gotschlich *et al.*, 2001) |
| H111-R | H111*cepR*::Km mutant of H111, Km$^R$ | (Huber *et al.*, 2001) |
| H111-I/R | marker less deletion mutant of *cepI*, *rsaM* and *cepR*, genotype: cepI/R::FRT | A. Carlier |
| H111ΔpC3 | *B .cenocepacia* H111, pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| H111pC3del1 | *B. cenocepacia* H111, partial pC3 deletion mutant | Kirsty Agnoli unpublished |
| H111ΔpC3/pC3$_{K56-2}$ | *B. cencocepacia* H111, pC3 deletion mutant complemented with pC3 from K56-2 | Kirsty Agnoli unpublished |
| H111ΔpC3/pRG930 | pRG930 cosmid containing H111 insert in *B. cencocepacia* H111, pC3 deletion mutant | This study |
| H111-*purD*::Tn*5* | H111::Tn5 insertional mutant, Km$^R$ | (Köthe, 2004) |
| H111-*purF*::Tn*5* | H111::Tn5 insertional mutant, Km$^R$ | (Köthe, 2004) |
| H111-*purL*::Tn*5* | H111::Tn5 insertional mutant, Km$^R$ | (Köthe, 2004) |
| H111-*purA*::Tn*5* | H111::Tn5 insertional mutant, | (Köthe, 2004) |

| | | |
|---|---|---|
| | $Km^R$ | |
| H111-*aroK*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*ilvC*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*cysI1*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*cysB2*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*ahcY*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*hisG*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*hisH*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*trpA*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*trpB*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*trpF*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*gatA*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*lon*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*rsaM*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*pyrD*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Köthe, 2004) |
| H111-*rsuA*::Tn*5* | H111::Tn5 insertional mutant, $Km^R$ | (Huber *et al.*, 2002) |
| K56-2 | CF isolate, ET12 strain | (Mahenthiralingam *et al.*, 2000) |
| J2315 | CF isolate, ET12 lineage strain | (Govan *et al.*, 1993) |
| MCO-3 (LMG24308) | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| MCO-3ΔpC3 | *B .cenocepacia* MCO-3 (LMG24308), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| H12424 (LMG24507) | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| H12424ΔpC3 | *B .cenocepacia* H12424 (LMG24507), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| **Other Bcc strains** | | |
| *B. cepacia* R18194 | Forest soil, Trinidad | (Stanier *et al.*, 1966) |
| *B. stabilis* R-6281 | Water outlet, Germany | (Gotschlich *et al.*, 2001) |
| *B. dolosa* LMG21820 | CF isolate, United Kingdom | (Vermis *et al.*, 2004) |
| *B. ambifaria* LMG17828 | Corn roots, USA | (Coenye *et al.*, 2001) |
| *B. multivorans* LMG18822 | CF isolate, Canada | (Vandamme *et al.*, 1997) |

| | | |
|---|---|---|
| *B. vietnamiensis* LMG10929 | *Oryza sativa* rhizosphere, Vietnam | (Gillis, 1995) |
| *B. pyrrocinia* LMG21822 | Cornfield soil, USA | (Vandamme *et al.*, 1997) |
| *B. anthina* LMG20983 | CF isolate, United Kingdom | (Vandamme *et al.*, 2002) |
| *B. lata* 383 LMG 22485T | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| *B. ubonensis* LMG20358 | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| *B. pyrrocinia* LMG14191 | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| *B. ambifaria* AMMD LMG19182 | Soil isolate, prototroph | BCCM/LMG Bacteria Collection |
| *B. diffusa* LMG24065 | CF-isolate, USA | BCCM/LMG Bacteria Collection |
| *B. multivorans* LMG18825 | CF isolate | BCCM/LMG Bacteria Collection |
| *B. metallica* LMG24068 | CF-isolate, USA | BCCM/LMG Bacteria Collection |
| *B. contaminans* LMG23361 | CF-isolate, Spain | BCCM/LMG Bacteria Collection |
| *B. seminalis* LMG24067 | CF-isolate, USA | BCCM/LMG Bacteria Collection |
| *B. latens* LMG24064 | CF-isolate, Italy | BCCM/LMG Bacteria Collection |
| *B. arboris* LMG24066 | CF-isolate, USA | BCCM/LMG Bacteria Collection |
| *B. stabilis* LMG7000 | CF-isolate, Sweden | BCCM/LMG Bacteria Collection |
| *B. lata 383*ΔpC3 | *B. lata* 383 (LMG22485T), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. ubonensis* LMG20358ΔpC3 | *B. ubonensis* (LMG20358), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. vietnamiensis* LMG10929ΔpC3 | *B. vietnamiensis* (LMG10929), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. anthina* LMG20983ΔpC3 | *B. anthina* (LMG20983), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. pyrrocinia* LMG14191ΔpC3 | *B. pyrrocinia* (LMG14191), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. ambifaria* LMG19182ΔpC3 | *B. ambifaria* (LMG19182), pC3 deletion mutant | (Agnoli *et al.*, 2012) |
| *B. diffusa* LMG24065ΔpC3 | *B. diffusa* (LMG24065), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. multivorans* LMG18825ΔpC3 | *B. multivorans (*LMG18825ΔC3), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. metallica* LMG24068ΔpC3 | *B. metallica* (LMG24068), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. contaminans* LMG23361ΔpC3 | *B. contaminans* (LMG23361), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. seminalis* LMG24067ΔpC3 | *B. seminalis (*LMG24067), pC3 deletion mutant | Kirsty Agnoli unpublished |

| | | |
|---|---|---|
| *B. latens* LMG24064ΔpC3 | *B. latens (*LMG24064), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. arboris* LMG24066ΔpC3 | *B. arboris* (LMG24066), pC3 deletion mutant | Kirsty Agnoli unpublished |
| *B. stabilis* LMG7000ΔpC3 | *B. stabilis* (LMG7000), pC3 deletion mutant | Kirsty Agnoli unpublished |
| Plasmids | | |
| pRG930 | 16.1-kb cosmid, derived from pGV910, $Sm^r/Sp^r$ | (Vandeneede *et al.*, 1992) |
| pRG930-*purD* | pRG930 containing *purD* gene $Sm^r/Sp^r$ | This study |
| pRG930-*purF* | pRG930 containing *purF* gene $Sm^r/Sp^r$ | This study |
| pRG930-*purL* | pRG930 containing *purL* gene $Sm^r/Sp^r$ | This study |
| pRG930-*pyrD* | pRG930 containing *pyrD* gene $Sm^r/Sp^r$ | This study |
| pRG930-*aroK* | pRG930 containing aroK gene $Sm^r/Sp^r$ | This study |
| pRK600 | CmR; ColE1*oriV*; RK2-Mob+ RK2-Tra+; helper plasmid for conjugative transfer | (Kessler *et al.*, 1992) |
| pGEM-3Zf(+) | $Ap^r$, multicopy cloning vector | Promega |
| pSB403 | $Tc^r$, *lux*RI' ::*lux*CDABE transcriptional fusion, bioluminescent AHL sensor plasmid | (Winson *et al*. 1998a) |
| pUTmini-Tn*5* Km2-*lux*CDABE | $Km^r$, delivery vector for miniTn*5*Km2-*lux*CDABE | (Winson *et al*. 1998b) |
| pCR2.1-TOPO | $Ap^r$, $Km^r$, cloning vector | Invitrogen |
| pUC19 | Cloning vector, $Ap^r$, *lacZ* | Invitrogen, Carlsbad, USA |
| | | |
| **Animals** | | |
| *Caenohabditis elegans* | Bristol N2 wild type | *Caenohabditis* Genetic Center, University of minnesota, USA |
| *Galleria mellonella* | Wild type | Fischerei Brumann |
| *Drosophila melanogaster* | Canton S wild type | This lab |

## 2.2 Table 2: Primers used in this study.

| Primer Name | Sequence 5‘ → 3‘ | Description |
|---|---|---|
| **pRG930_seq_rv** | ACG TTT CAG TTT GCT CAT GG | Sequencing primer pRG930 |
| **pRG930_seq_fw** | GGT CCT GCA ACT TTA TCC G | Sequencing primer pRG930 |
| **Kan res-v** | ACC GAG GCA GTT CCA TAG G | Mini-Tn*5* Km2-*lux*CDABE |
| **Kan res-r** | ATG AGC CAT ATT CAA CGG G | Mini-Tn*5* Km2-*lux*CDABE |
| **M13v** | GTA AAA CGA CGG CCA G | Sequencing primer/pCR2.1TOPO |
| **M13r** | CAG GAA ACA GCT ATG AC | Sequencing primer/pCR2.1TOPO |
| **ARB2** | GGC CAC GCG TCG ACT AGT AC | Arbitrary-PCR, round 2 |
| **ARB6** | GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC | Arbitrary-PCR, round 1 |
| ***lux*Cext2** | AGT CAT TCA ATA TTG GCA GG | Arbitrary-PCR, round 1 |
| ***lux*Cint2** | GGA TTG CAC TAA ATC ATC CAC | Arbitrary-PCR, round 2 |

## 2.3 Non-mammalian pathogenicity models

### 2.3.1 *C. elegans* infection model

All the pathogenicity assays which were done in this work were done by using the slow killing assay was performed as described by Köthe (Koethe *et al.*, 2003). The *C. elegans* strain used for the studies was the *C. elegans* N2 Bristol which was obtained from the *Caenorhabditis* Genetics Centre (CGC, University of Minnesota, USA). The bacterial strain of interest was grown on nematode growth medium (NGM). After the bacteria had formed a lawn around 20 to 40 worms were transferred onto the plate. The worms ingest the bacteria which will colonize the nematode intestine and after a time period of 2 to 5 days they kill their host. For all the assays *C. elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis, USA), had been used.

### 2.3.2 Media and buffer for *C. elegans*

**Nematode growth medium I (NGMI)** (**Hope**, 1999)

51 mM NaCl, 0.25% (w/v) tryptone, 1.7% (w/v) agar

Plus additives

**Nematode growth medium II (NGM II) (Tan *et al.*, 1999a)**

51 mM NaCl, 0.35% (w/v) bactopeptone, 1.7% agar

Plus additives

**Additives for NGM I/NGM II (final concentration)**

25 mM KPO4 buffer [pH 6.0], 1 mM CaCl2, 1 mM MgSO4, 2 µg/ml uracil, 5 µg/ml cholesterol, 50 µg/ml nystatine

All the supplements were autoclaved sepperatly and added to hand warm NGMI or II medium except the Nystatine solution which was sterile filtrated

KPO4 buffer: 0.8 M KH2PO4, 0.2 M K2HPO4 [pH 6.0]

*Nystatine solution*: 10 mg/ml in 1:1 of 100% ethanol and 7.5 M ammonium acetate

**M9 buffer**

22 mM KH2PO4, 42 mM Na2HPO4, 85 mM NaCl, 1 mM MgSO4

### 2.3.3 Cultivation and stock conservation of *C. elegans*

*C. elegans* were kept under lab conditions (20°C) on NGMI plates which were overgrown with the food strain E. coli OP50. Under this conditions they can kept up to three weeks. To prepare food plates 150 µl of a fresh *E. coli* OP50 culture was dropped on NGMI plates and incubated at 37°C overnight. The plates can be kept for several weeks at 4°C in the fridge. For conservation small agar pieces from old nematode plates were transferred onto the fresh NGMI plates every week.

### 2.3.4 Egg preparation

To obtain the same precondition for all the nematode for the pathogenicity experiment which is very important, the *C. elegnas* should be in the same L4 larval stage. This could be done with an egg preparation by using a bleaching method. Two days after the nematodes were transferred onto the fresh plates they laid a large number of eggs. These can now be rinsed four times with 1 ml of sterile water and the nematode egg suspension was distributed into three 1.5 ml tubes. To every tube 500 µl of the hypochlorite solution was added (600 µl sterile MQ water, 500 µl 12% sodium hypochlorite and 400 µl 6M NaOH). The tubes were inverted several times for 8 to 10 min. After a centrifugation step (1 min, 3200 rpm in an Eppendorf table centrifuge) the supernatant was discarded and the pellets centrifuged again after washing them with 1 ml sterile water. The pellets were suspended with 100 µl M9 buffer and combined into one tube. The eggs were transferred to new *E. coli* OP50 seeded NGMI plate.

### 2.3.5 Pathogenicity assay

At the same day of the egg preparation, the bacterial strain to be tested were inoclulated in 5 ml LB medium and incubated overnight at 37°C. The following day 6 well plates (Greiner, Frickenhausen, Germany) containing NGMII medium were inoculated with 150 µl culture per well. After another incubation step at 37°C the L4 stage worms can be rinsed from the NGMI plate and approximately 20 to 40 worms were transferred to the plate containing the bacterial lawns. To keep the plates in the dark, they were covered with foil an incubated at 20°C. The nematodes were counted in 1- day intervals using a Zeiss Stemi SV binocular (Zeiss, Göttingen, Germany). For some experiments the so called pathogenicity score was used. This score is composed of the total number of points given due to different criteria. One point is given if worms are sick at day two of the experiment, another if more than 30% were dead at the 2$^{nd}$ day and the third if the total number of worms is less than 50 at day number five. A score of zero indicates a non-pathogenic and three a very pathogenic strain.

### 2.3.6 *G. mellonella* pathogenicity assay

Assays were carried out essentially as described by (Jander *et al.*, 2000; Seed & Dennis, 2008; Uehlinger *et al.*, 2009). Fifth instar caterpillars were obtained in fishing equipment store (Fischerei Brumann, Zürich) and stored at 14°C in wooden shavings. The larvae were used no later than 2 to 3 weeks after the day of purchase. The strains of interest were grown overnight in LB and diluted in the morning of the experimental day 1:100 in 30 ml fresh LB and cultivated again at 37°C until an optical density at 600 ($OD_{600}$) of 0.4 to 0.7 which indicated the logarithmic growth phase. The cultures were centrifuged and the pellet was resuspended with a physiological buffer of 10 mM $MgSO_4$ to an $OD_{600}$ of one. For the *Burkholderia* strains 1 to 2 dilutions were made (10 mM $MgSO_4$) to an $OD_{600}$ of 0.0625 and for *Pseudomonas* $LD_{50}$ experiment 1 to 10 to $OD_{600}$ of 0.000001. After preparing the dilutions, 10 µl aliquots were injected with a 1 ml syringe (BD Plastipak, Madrid, Spain) containing a 27G x 7/8" needle (Rose GmbH, Trier, Germany) into the hindmost proleg. For faster handling the, larvae were taped on the bench and the area around the proleg was disinfected with a cotton swap which was soaked in ethanol. For *Burkholderia* experiments 10 larvae were used per trail and for the *Pseudomonas* $LD_{50}$ experiment 6 per dilution and trail. Each experiment was done at least in triplicate. As a control 10 larvae were injected with 10 µl $MgSO_4$ buffer (to exclude contaminations). The infected larvae were kept in petri dishes and incubated at 30°C in the dark. Dead larvae were counted after 20, 24, 40, 48 and 72 hours after infection.

### 2.3.7 *D. melanogaster* pathogenicity assays

*D. melanogaster* feeding or pricking assays were performed as described by Apidianakis (Apidianakis & Rahme, 2009). In this study the *D. melanogaster* Canton S wild type strain was used. The bacterial strain of interest was grown over night ml was harvest and resuspended in 1 ml $MgSO_4$ and 15 flies per strain were injected with a very thin needle into the thorax (pricking experiment). For the feeding 2 ml of an overnight culture were harvest and resuspended with 170 µl of an 5 % sucrose solution and dropped on a filter disc which is on top of an fly vial containing 10 ml agar-sucrose solution (2 % agar, 5 % sucrose). Ten individuals were transferred into each vial. Depending on the experiment (pricking, feeding) and the bacteria used (*Pseudomonas*, *Burkholderia*) the dead flies were counted after hours to days post infection.

### 2.3.8 Media for *D. melanogaster*

Cornmeal, sucrose and Yeast medium

This medium was done like described by Lewis (Lewis, 1960) with some modifications.

0.79% (w/v) agar, 2.75% (w/v) yeast, 2.6% (w/v) cornmeal, 321 mM sucrose and 50 µg/ml nystatine (nystatine solution see *C. elegans* media)

First agar was solved in boiled water then cornmeal, sugar and yeast was added under constant stirring. The medium was cooks for minimum 30 min. After cooling (hand warm) the nystatin solution was added and the vials (50 ml Falcon tubes) were filled 2-3 mm high with the cornmeal medium. It can be stored for several weeks at 4°C.

**Sucrose agar**

2.4% (w/v) agar and 5% (w/v) sucrose

The sucrose agar can be autoclaved under normal conditions.

### 2.3.9 Stock conservation of *D. melanogaster*

For the stock conservation the fruit flies were transferred every two weeks into new vials containing food. They were stored in the lab at room temperature at around 20°C.

### 2.3.10 *D. melanogaster* feeding assay

The *D. melanogaster* feeding assay was performed as described by (Chugani *et al.*, 2001). Two ml bacterial cells from an overnight culture were harvest by centrifugation and

resuspended in 170 µl of a 5% sucrose solution. The bacterial solution was added to a Whatman filter disk which completely covered the sucrose agar surface which is at the bottom of a fly culture vial. Ten *D. melanogaster* Canton S flies with an age between three to five days were added after a starving time of around 5 h to each vial. The vials were closed with cotton and incubated at 26°C. For a constant humidity the vials were put in a closed box containing wet tissues. Dead flies were counted every 24 h.

### 2.3.11 *D. melanogaster* pricking assay

The *D. melanogaster* pricking assay was performed as described by (Apidianakis & Rahme, 2009) Fly culture vials were filled with around 8 ml of the sucrose agar solution. On top a filter disk (Whatman paper) was added that completely covered the surface. The filters were soaked with 200 µl of 5% sucrose solution which served as food for the flies. One ml of overnight cultures of bacteria were pelleted by centrifugation and resuspended in 1 ml 10 mM $MgSO_4$ solution. For the infection process the flies were anesthesized with ether and a Thungsten stainless steel needle with diameter of approximate 0.01 mm at the tip was dipped into the prepared bacterial solution before pricking into the thorax of the flies. For each bacterial strain 15 flies were used and if more died because of the injection, the experiment was not counted. As control the needle was dipped into $MgSO_4$ buffer. The vials were incubated into a wet box at 26°C and the flies were counted at 2h intervals at the 2$^{nd}$ day of infection for *Pseudomonas* and after every 24h for *Burkholderia* until all flies were dead.

## 2.4 Construction of a cosmid library of *B. cenocepacia* H111 by using the pRG930 cosmid

### 2.4.1 Total DNA extraction of *B. cenocepacia*

Chromosomal *Burkholderia* DNA was isolated as described (Better *et al.*, 1983). For the extraction 5 ml of an overnight culture was harvest by centrifugation at 5000 rpm for 8 min. The pellet was resuspended in 2 ml TE-buffer (50 mM Tris, 20 mM EDTA, pH8) followed by adding 2.5 ml of 2% sarcosyl (Sigma, Buchs, Switzerland). To remove proteins 0.5 ml pronase (5mg/ml, Sigma, Buchs, Switzerland), which was first activated at 37°C for 30 min, was added and incubated at 37°C until the solution cleared (10 - 30 min). After vortexing for 2 min at maximal speed the same amount of phenol/chloroform/*iso*-amyl alcohol (25:24:1) was added to the tube. The solution was mixed carefully by converting the tube several times and the supernatant was transferred to a new tube with catted pipet tips after a centrifugation step (5 min, 5000 rpm) and the same amount of chloroform (Sigma, Buchs, Switzerland) was

added followed by a second centrifugation step. The DNA was precipitated by adding NaCl to the supernatant (stock solution: 5 M, final concentration: 0.2 M) and an equal volume of 100% EtOH. The precipitated DNA was carefully fished out with a plastic loop, washed by dipping the loop into 70 % of ice cold EtOH. After air-dry, the DNA was solved in 500 – 100 µl MQ (depending on the working concentration needed). To remove the RNA 10 µl of RNase (10 mg/ml) was added.

### 2.4.2 Partial digestion of the extracted H111 DNA

For the partial digestion two master mixes were prepared (one with 130 µl DNA, 25 µl Puffer 10 x, 85 µl MQ and one with 1.5 µl EcoRI (New England Biolabs), 1 µl buffer 10x, 7.5 µl MQ) and pre warmed at 37°C. The pre warmed master mixes were pooled and incubated at 37°C. Every minute (started at minute two) 30 µl of the digestion reaction was taken and directly frozen in dry ice to stop the reaction. After all samples were taken except the one which should be totally digested (as a control) the reaction was finally stopped by heat inactivation (65 – 70°C during 15 – 20 min). Five 5 µl of each tube from the digestion were loaded on a gel to decide which reactions can be taken for the library because the insert should have a size between 20 and 40 kilo bases. Normally five tubes from minute 2, 3, 4, 5 and six looked good and they could be pooled.

### 2.4.3 Pooling of the partial digested H111 DNA

For the precipitation of the DNA 0.1 volume of 3 M NaAcetate was added (final concentration: 0.3 M). Then two times the volume of 100 % EtOH was added and well vortexed. The sample were centrifuged (15 min at maximal speed) and 70 % EtOH was added carefully to prevent not to destroy the pellet followed by another centrifugation step (5 min at maximal speed). After the pellet was air dried it was solved in 40 µl MQ.

### 2.4.4 Extraction of the cosmid vector

For the extraction of the pRG930 cosmid (Vandeneede *et al.*, 1992) vector 200 ml of an overnight culture was harvest. The extraction itself was done witch a QUIAGEN Midiprep kit according the manufactures' protocol. The DNA pellet was redissolved in 100 µl MQ. 30 µl of the vector was digested with 0.5 µl of the restriction enzyme EcoRI (New England Biolabs) 5 µl buffer and 14 µl MQ during four to six hours at 37°C. To prevent religation of the vector

the 5'-posphates were removed by adding 2 µl SAP (shrimp alkaline phosphatase) followed by incubation for 1 h at 37°C. Then the enzyme was inactivated by heating the reaction up to 65°C for 20 min.

### 2.4.5 Ligation of the pRG930 with the partial digested DNA

For the ligation of the cosmids with the vectors a ratio of 1:1 was used whereby the final amount of the reaction was 15 µl (if the master mix was bigger the rest was removed by evaporation). The reaction was incubated overnight at 18°C.

### 2.4.6 Packaging and tittering the cosmid packaging reaction

Packaging and titering the cosmid packaging reaction into *E. coli* HB101 cells was done by using the Gigapack III Gold Packaging reaction Kid from Invitrogen as described in the instruction manual. The only modification of the protocol was, that the dilution step 7 of the titering reaction was skipt(because the efficiency is not extreamly high anyway). Cosmids were transferred to *Burkholderia via* triparental mating.

# 3 Results and Discussion

## 3.1 Investigations on the pathogenicity of *P. aeruginosa* and Bcc strains using multiple infection hosts

### 3.1.1 *C. elegans* pathogenicity assay

Due to its small size, the sequenced genome, massive production of offspring's (Ewbank, 2002) and the short generation time of less than three days at 25°C (Byerly *et al.*, 1976), the nematode *C. elegans* is a cheap and easy model organism to screen libraries of mutants or drug candidates. The transparency of the organism allows following bacterial colonization of the gut by microscopy using confocal laser microscopy. In combination with fluorescently tagged *B. cenocepacia* J2315 wild-type and ACP mutant strains (acyl carrier protein which plays a role in the biosynthesis of fatty acids) the colonization behaviour of the strains was investigated. The ACP mutant was found to be less virulent and showed also fewer bacteria in the intestinal tract compared to the wild-type. The microscopic pictures showed a reduced ability to colonize the *C. elegans* gut (Sousa *et al.*, 2008).

By using the *C. elegans* slow killing assay a Tn*5*-transposon library of *B. cenocepacia* H111, which contained around 5'500 mutants, was screened. Because it was known that quorum sensing controls virulence in many pathogenic bacteria, the library was screened for attenuated mutants which produced the signal molecule to find genes responsible for pathogenicity but are not affected in QS. 22 attenuated mutants were detected by this screen. Surprisingly, none of the mutants was defective in a gene encoding classical virulence factors. Phenotypic characterization of these mutants revealed that protease activity, EPS-production, siderophore production and expression of the nematode protein AidA was altered in some but not all of the strains. The fact that all mutants were able to grow as fast as the wild-type in LB or PIA medium but not in minimal media ABC or ABG was surprising. The inactivated genes were *purA, purD, purL* and *purF* (purine pathway), *aroK* (shikimat pathway), *cysI1* and *cysB2* (cysteine pathway), *pyrD* (pyrimidine pathway), *hisG* and *hisH* (histidine pathway), *trpA, trpB* and *trpF* (tryptophane pathway), *ilvC* (isoleucine valine pathway), *ahcY, rsuA, gatA, yciL* and *Ion*. However, because temperature effects, food avoidance and host-specific factors cannot be excluded, the attenuated strains which were also tested in two additional animal models, namely *D. melanogaster* and *G. mellonella*.

### 3.1.2 *G. mellonella* pathogenicity assay

The second non-mammalian animal host used as infection host is the greater wax moth larvae *G. mellonella*. The big advantage of using this animal is that a well-known dosage of bacteria can be injected. Due to this reason determination of the $LD_{50}$ is possible. Another advantage is that the strain of interest can be tested at temperatures up to 37°C unlike *C. elegans* and *D. melanogaster*. Like other non-mammalian hosts the experiments are quite cheap, no permission is required and the experiments need not a lot of space. A disadvantage of this model is that the genome of *G. mellonella* has not been sequenced and no genetic tools are available to manipulate this organism. The experiments need more time when compared to the *C. elegans* and *D. melanogaster* models and therefore screenings of libraries are not possible with this host. Therefore, only the auxotrophic mutants from the Tn*5*-transposon library, which were attenuated in *C. elegans* and *D. melanogaster*, were also tested for their pathogenicity in *G. mellonella*. With the Exception of the *ilvC* mutant all other mutants (*purD, purL, purF, aroK* and *pyrD*) showed reduced virulence.

### 3.1.3 *D. melanogaster* pathogenicity assay

*D. melanogaster* is like *C. elegans* another useful tool to test the virulence of different bacterial strains. The advantages are quite similar to those of the nematode model. *D. melanogaster* has a sequenced genome, it is a small organism which needs not a lot of space and its generation time is short with a lot of offspring's. One of the advantages to work with this animal is that both chronic and acute infections can be investigated. The pricking assay is similar to the infection assay of *G. mellonella* and the feeding assay to chronic infection of *C. elegans*. Hence, the fly is a perfect tool to compare acute and chronic infections. The strains chosen to be tested in the fruit fly *D. melanogaster* Canton S were the same as for the *G. mellonella* infection model (Uehlinger *et al.*, 2009). The H111-I, H111-R and H111-I/R QS mutants of *B. cenocepacia* H111 were also tested. The results from the pricking assays were compared with those of the *G. mellonella* infection study to assess if host-specific factors play a role. Strains which killed the flies in less than 200 hours were categorized as pathogenic, such with more than 30% of survivors at this time point as attenuated. *B. stabilis* R6281, *B. anthina* LMG20983 and *B. multivorans* LMG1822 showed attenuation when tested in *G. mellonella (*Uehlinger *et al.*, 2009*)*. For *B. anthina* LMG20983 and *B. multivorans* LMG1822 the same trend could be observed when tested with the *D. melanogaster* pricking model, were more than 70% of the flies survived after 200 hours (figure 4B). This was not the case for *B. stabilis* R6281 were all flies where dead after less than 150 hours (figure 4A). The opposite

was observed for the strains *B. cenocepacia* J2315, *B. dolosa* LMG21820 and *B. ambifaria* LMG17828, which showed to be fully attenuated when tested in the *D. melanogaster* pricking assay (figure 4B), but highly pathogenic to the *G. mellonella* larvae (Uehlinger *et al.*, 2009). *B. cenocepacia* H111, *B. cenocepacia* 56-2, *B. cepacia* R18194, *B. vietnamiensis* LMG10929 and *B. pyrrocinia* were highly virulent in the *D. melanogaster* and *G. mellonella* model (figure 4A (Uehlinger *et al.*, 2009)). Compared to the wild type no difference in virulence was observed with the QS mutants. It seems that QS does not play an essential role in pathogenicity in acute infection models like *D. melanogaster* pricking (figure 4C) or *G. mellonella* while in the chronic *C. elegans* model it did play an important role. We could also show that the attenuation reported for the *cepI* mutant in *G. mellonella (*Uehlinger *et al.*, 2009*)* resulted from a partial deletion of pC3. Experiments with a newly constructed *cepI* mutant showed no difference when compared to the wild type.

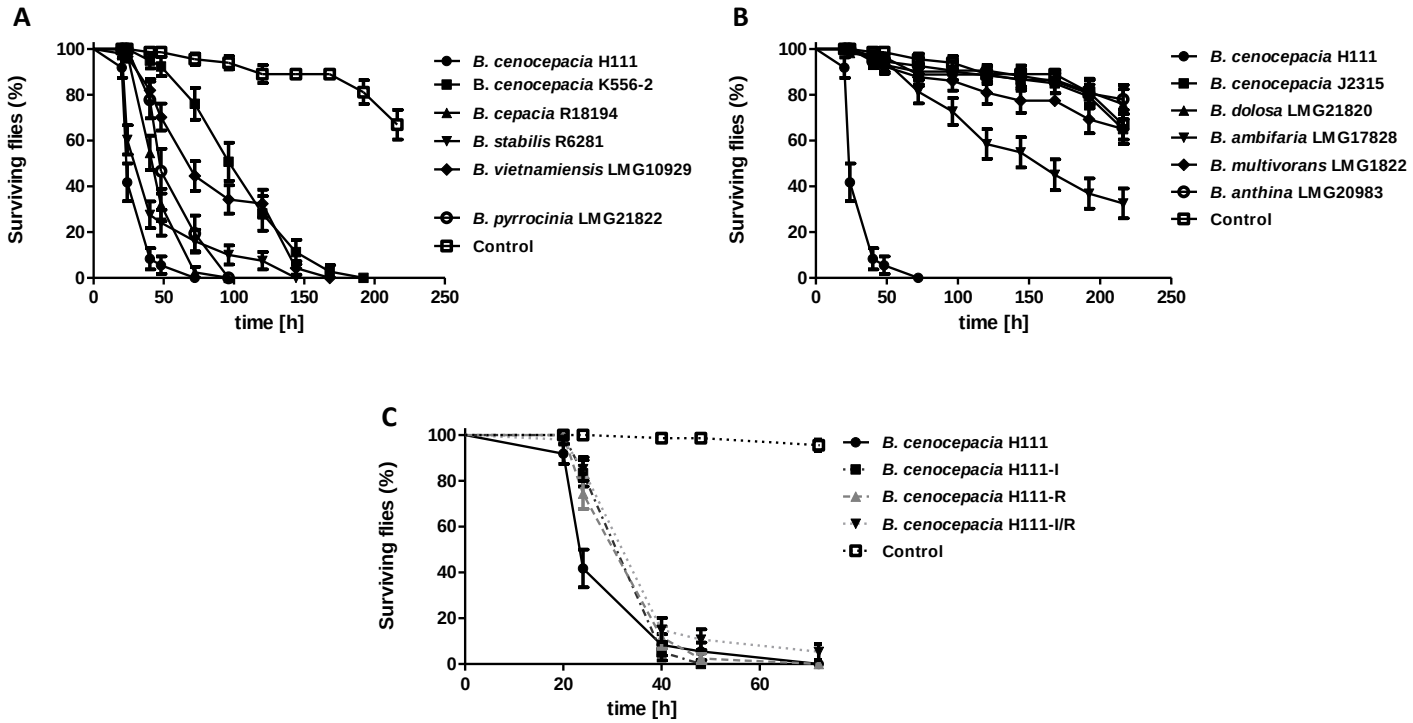


**Figure 4:** *D. melanogaster* pricking assay with different Bcc strains. Figure **A**; strains which showed no attenuation; these strains were as pathogenic as *B. cenocepacia* H111. Within less than 200 h, all flies were killed: Figure **B**; the Bcc strains with low levels of pathogenicity where more than 30% survived after more than 200 h and **C** the *cepI*, *cepR* and *cepI/R* of *B. cenocepacia* H111.

The feeding assay did not work well for Bcc strains (data not shown); (Castonguay-Vanier *et al.*, 2010). The reason for that is that Bcc strains were not pathogenic enough in this experiment relative to *P. aeruginosa*. The pricking assay on the other hand turned out to be a useful tool for determining the virulence of Bcc strains. Because every single fly has to be pricked by hand to inoculate the animal it is for time reasons not possible to screen whole libraries with thousands of mutants, but it is possible to test a few hundred strains. This is much more than what can be done with *G. mellonella*. It was therefore not possible to screen the entire Tn*5*- library using the *D. melanogaster* pricking assay, but all the mutants, which were attenuated in the *C. elegans* assay, were tested. Six out of the mutants identified in the *C. elegans* screen were also less virulent in *D. melanogaster*: *ilvC, purD, purL, purF, aroK* and *the pyrD* mutants represent this group.

## 3.2 Novel drug targets from the *B. cenocepacia mutant screen*

*Burkholderia cenocepacia* H111, a strain isolated from a cystic fibrosis patient, has been shown to effectively kill the nematode *Caenorhabditis elegans*. We used the *C. elegans* model of infection to screen a mini-Tn*5* mutant library of *B. cenocepacia* H111 for attenuated virulence. Among the approximately 5,500 *B. cenocepacia* H111 random mini-Tn*5* insertion mutants that were screened, 22 showed attenuated virulence in *C. elegans*. None of the mutated genes coded for the biosynthesis of classical virulence factors such as extracellular proteases or siderophores. Instead, we found several mutants with deletions in metabolic and regulatory genes. Within the attenuated mutants, four pathways involved in amino acid production were disrupted. These were the cysteine, valine and isoleucine, histidine and shikimate pathways. The shikimate pathway is responsible for the production of chorismate, which is the precursor of aromatic amino acids including tyrosine, phenylalanine and trypthophan (Meibom & Charbit. 2010). In addition to these, auxotrophic mutants disrupted in the purine and pyrimidine pathways were isolated. Three regulatory mutants were also identified, and the mutants were phenotypically characterised for the production of classical VFs (virulence factors), to test whether the observed attenuation in virulence was an indirect result of the disrupted gene on a known VF.

In cancer therapy purine inhibitors are in use (Bertino *et al.*, 2011; Issaeva *et al.*, 2010). We next tested whether *Burkholderia* sp. can be inhibited by these compounds as well. An inhibition assay with 6-thioguanine (Bertino *et al.*, 2011; Issaeva *et al.*, 2010) showed no effect. Possibly *B. cenocepacia* uses a different uptake system, or the purine structure is too different when compared to the human system. Other interesting pathways found in this study, which showed attenuation in all organisms tested, was the one involved in pyrimidine biosynthesis (*pyrD* gene) and the shikimat pathway (*aroK* gene) which codes for shikimat kinase. The shikimat pathway is present and conserved in bacteria, fungi algae and plants and is absent in mammals (Kishore & Shah, 1988; Roberts *et al.*, 1998). For bacteria it is essential for the synthesis of folic acid, aromatic amino acids and ubiquinone (Parish & Stoker, 2002). Hence, the shikimat pathway has been suggested to be an excellent target for the development of new antibacterial agents (Han *et al.*, 2007; McConkey, 1999; Norris *et al.*, 2009). A compound already in use which targets the skikimat pathway is the herbicide glyphosate, which is well known as Roundup from Monsanto. This compound inhibits the 5-enoylpyruvyl shikimat phosphate synthase (Han *et al.*, 2007). Glyphosate has been shown to interfere with

tyrosine, phenylalanine, tryptophane, phosphoenolpyruvate anabolic pathways and the tricarboxylic acid cycle of various bacteria, including *Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis and Bradyrhizobium japonicum* (Fischer *et al.*, 1986; Norris *et al.*, 2009; Zablotowicz & Reddy, 2004). An inhibition of the growth of *B. cenocepacia* H111 could also show by using glyphosate but it is not a lethal inhibition.

# Identification of *Burkholderia cenocepacia* H111 virulence factors using non-mammalian infection hosts.

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# Identification of *Burkholderia cenocepacia* Strain H111 Virulence Factors Using Nonmammalian Infection Hosts

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***Burkholderia cenocepacia* H111, a strain isolated from a cystic fibrosis patient, has been shown to effectively kill the nematode *Caenorhabditis elegans*. We used the *C. elegans* model of infection to screen a mini-Tn*5* mutant library of *B. cenocepacia* H111 for attenuated virulence. Of the approximately 5,500 *B. cenocepacia* H111 random mini-Tn*5* insertion mutants that were screened, 22 showed attenuated virulence in *C. elegans*. Except for the quorum-sensing regulator *cepR*, none of the mutated genes coded for the biosynthesis of classical virulence factors such as extracellular proteases or siderophores. Instead, the mutants contained insertions in metabolic and regulatory genes. Mutants attenuated in virulence in the *C. elegans* infection model were also tested in the *Drosophila melanogaster* pricking model, and those also attenuated in this model were further tested in *Galleria mellonella*. Six of the 22 mutants were attenuated in *D. melanogaster*, and five of these were less pathogenic in the *G. mellonella* model. We show that genes encoding enzymes of the purine, pyrimidine, and shikimate biosynthesis pathways are critical for virulence in multiple host models of infection.**

The *Burkholderia cepacia* complex (Bcc) consists of 17 closely related, but phenotypically diverse, bacterial species. Strains of the Bcc have been isolated from soil, the rhizospheres of plants, groundwater, industrial settings, hospital environments, and infected humans and animals. Some strains could be valuable for biotechnology, as they can be used for bioremediation, as biological pest control agents, and for plant growth promotion. Conversely, some strains from the Bcc can act as problematic opportunistic pathogens in patients suffering from cystic fibrosis (CF) and also in immunocompromised individuals (1–4). *B. cenocepacia* and *Burkholderia multivorans* are currently the Bcc species most frequently isolated from clinical samples (1, 4). In CF patients these species can cause serious infections, leading to rapid decline in lung function and an often fatal pneumonia (known as "cepacia syndrome") (5, 6).

Over the past decade, substantial progress has been made in identifying and characterizing the virulence determinants and infection mechanisms of Bcc strains (7). These studies used different Bcc strains and various infection models. Since strain-to-strain variability and the infection model used have a tremendous effect on the outcome of pathogenicity assays, knowledge of the importance of individual virulence factors in different infection hosts is sparse (7, 8).

In this study, we screened a *Burkholderia cenocepacia* H111 random insertion mutant bank for attenuated virulence in *Caenorhabditis elegans*. To exclude experimental and host-specific factors, other nonmammalian animal models were used to test those mutants which showed attenuation in pathogenicity to *C. elegans*. The first was the fruit fly, *Drosophila melanogaster*, into which bacteria were injected by pricking with a needle (9, 10). Those mutants that were also attenuated in *D. melanogaster* were further tested using the greater wax moth larva *Galleria mellonella*, which allows injection of a precise dosage of bacteria (8, 11). We checked mutants for the production of AidA, which is known to play a role in pathogenicity to *C. elegans* (8, 12), as well as for other previously identified virulence factors, including production of acyl-homoserine lactone (AHL) signal molecules, siderophores, proteases, and polysaccharides.

## MATERIALS AND METHODS

**Organisms and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, bacteria were grown at 37°C in modified Luria-Bertani (LB) broth (13) containing 5 g NaCl liter$^{-1}$. Solid media contained agar to a final concentration of 1.5%. Antibiotics were added as required at final concentrations of 20 μg ml$^{-1}$ gentamicin, 50 μg ml$^{-1}$ kanamycin, 10 μg ml$^{-1}$ tetracycline, 50 μg ml$^{-1}$ spectinomycin, 25 μg ml$^{-1}$ streptomycin, and 10 μg ml$^{-1}$ chloramphenicol. Growth of liquid cultures was monitored by measurement of optical density at 600 nm ($OD_{600}$) using an Ultraspec 3100 pro (Amersham Bioscience). Supplements were added to media to the following concentrations: L-histidine, L-cysteine, DL-tryptophan, and adenosine, 500 μM; guanine, adenine, adenosine, and inosine, 20 μg liter$^{-1}$. Killing assays were performed using *Caenorhabditis elegans* strain Bristol N2, which was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, St. Paul, MN). Nematodes were maintained on NG agar (14) at 20°C with *Escherichia coli* strain OP50 as a food source (15). For the *D. melanogaster* pricking assay, the *D. melanogaster* Canton-S wild-type strain was used. The flies were kept at room temperature and fed with standard sucrose cornmeal (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/harvardfood.htm). *G. mellonella* was bought from Fischerei Brumann,

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**TABLE 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| *E. coli* | | |
| OP 50 | Food source strain for *C. elegans*, uracil auxotrophic | 15 |
| XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*$^q$ ZΔM15 Tn*10* (Tet$^r$)] | Stratagene |
| MT102 | *araD139* (*ara-leu*)*7679* Δ*lac thi hsdR* | Laboratory collection |
| *Pseudomonas putida* F117(pAS-C8) | Bioluminescent AHL biosensor, F117 with pBBR1MCS-5 carrying P*cepI*::*gfp*(ASV) P*lac*::*cepR*; Gm$^r$ | 77 |
| *Burkholderia cenocepacia* | | |
| H111 | CF isolate, Germany | 78 |
| H111-R | Km$^r$; *cepR*::Km mutant of H111 | 79 |
| H111 *purD* | Km$^r$; *purD*::Km mutant of H111 | This study |
| H111 *purF* | Km$^r$; *purF*::Km mutant of H111 | This study |
| H111 *purL* | Km$^r$; *purL*::Km mutant of H111 | This study |
| H111 *purA* | Km$^r$; *purA*::Km mutant of H111 | This study |
| H111 *aroK* | Km$^r$; *aroK*::Km mutant of H111 | This study |
| H111 *ilvC* | Km$^r$; *ilvC*::Km mutant of H111 | This study |
| H111 *cysI* | Km$^r$; *cysI*::Km mutant of H111 | This study |
| H111 *cysB* | Km$^r$; *cysB*::Km mutant of H111 | This study |
| H111 *ahcY* | Km$^r$; *ahcY*::Km mutant of H111 | This study |
| H111 *hisG* | Km$^r$; *hisG*::Km mutant of H111 | This study |
| H111 *hisH* | Km$^r$; *hisH*::Km mutant of H111 | This study |
| H111 *trpA* | Km$^r$; *trpA*::Km mutant of H111 | This study |
| H111 *trpB* | Km$^r$; *trpB*::Km mutant of H111 | This study |
| H111 *trpF* | Km$^r$; *trpF*::Km mutant of H111 | This study |
| H111 *gatA* | Km$^r$; *gatA*::Km mutant of H111 | This study |
| H111 *lon* | Km$^r$; *lon*::Km mutant of H111 | This study |
| H111 *rsaM* | Km$^r$; *rsaM*::Km mutant of H111 | This study |
| H111 *pyrD* | Km$^r$; *pyrD*::Km mutant of H111 | This study |
| H111 *rsuA* | Km$^r$; *rsuA*::Km mutant of H111 | 43 |
| *G. mellonella* | | Fischerei Brumann, Zürich |
| *C. elegans* Bristol N2 | | *Caenorhabditis* Genetics Center, University of Minnesota, Minneapolis |
| *D. melanogaster* Canton-S | | |
| Plasmids | | |
| pRK600 | Cm$^r$ *ori*ColE1 RK2-Mob$^+$ RK2-Tra$^+$, helper plasmid in triparental conjugations | 21 |
| pSB403 | Tc$^r$, *lux*RI′::*lux*CDABE transcriptional fusion, bioluminescent AHL sensor plasmid | 80 |
| pGEM-3Zf(+) | Ap$^r$, multicopy cloning vector | Promega |
| pUTmini-Tn*5* Km2-*luxCDABE* | Km$^r$, delivery vector for mini-Tn*5* Km2-*luxCDABE* | 81 |
| pCR2.1-TOPO | Ap$^r$, Km$^r$, cloning vector | Invitrogen |
| pRG930 | 16.1-kb cosmid, derived from pGV910, Sm$^r$/Sp$^r$ | 20 |
| pRG930-*purD* | pRG930 containing *purD* gene, Sm$^r$/Sp$^r$ | This study |
| pRG930-*purF* | pRG930 containing *purF* gene, Sm$^r$/Sp$^r$ | This study |
| pRG930-*purL* | pRG930 containing *purL* gene, Sm$^r$/Sp$^r$ | This study |
| pRG930-*pyrD* | pRG930 containing *pyrD* gene, Sm$^r$/Sp$^r$ | This study |
| pRG930-*aroK* | pRG930 containing *aroK* gene, Sm$^r$/Sp$^r$ | This study |
| pUC19 | Cloning vector, Ap$^r$, *lacZ* | Invitrogen, Carlsbad, CA |

Zurich, Switzerland, and kept in an incubator at 14°C. The larvae were used within 3 weeks of purchase.

**Transposon insertion mutagenesis.** The hybrid transposon (Table 1) was randomly inserted into the genome of *B. cenocepacia* H111 by triparental mating as described previously (24). Transconjugants were selected on LB medium containing kanamycin and tetracycline. These random insertion mutants were picked and grown in 150 μl LB medium in polypropylene microtiter plates (Nunc). For storage, 75 μl of 50% (vol/vol) glycerol was added and the plates were frozen at −80°C.

**DNA manipulations and sequence analysis of Tn*5* mutants.** Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (16). PCR was performed using TaKaRa rTaq DNA polymerase (TaKaRa Shuzo), as per the manufacturer's instructions. Plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen), and chromosomal DNA from *B. cenocepacia* was purified with the DNeasy tissue kit (Qiagen). DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen).

To ensure that the attenuated mutants bore only one transposon,

**TABLE 2.** Primers used in this study

| Primer | Sequence (5′→3′) | Description |
|---|---|---|
| pRG930_seq_rv | ACG TTT CAG TTT GCT CAT GG | Sequencing primer, pRG930 |
| pRG930_seq_fw | GGT CCT GCA ACT TTA TCC G | Sequencing primer, pRG930 |
| Kan res-v | ACC GAG GCA GTT CCA TAG G | Mini-Tn*5* Km2-*luxCDABE* |
| Kan res-r | ATG AGC CAT ATT CAA CGG G | Mini-Tn*5* Km2-*luxCDABE* |
| M13v | GTA AAA CGA CGG CCA G | Sequencing primer/pCR2.1TOPO |
| M13r | CAG GAA ACA GCT ATG AC | Sequencing primer/pCR2.1TOPO |
| ARB2 | GGC CAC GCG TCG ACT AGT AC | Arbitrary PCR, round 2 |
| ARB6 | GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC | Arbitrary PCR, round 1 |
| luxCext2 | AGT CAT TCA ATA TTG GCA GG | Arbitrary PCR, round 1 |
| luxCint2 | GGA TTG CAC TAA ATC ATC CAC | Arbitrary PCR, round 2 |
| Seq_O | CAC TTG TGT ATA AGA GTC AG | Sequencing primer, o end of the mini-Tn*5* Km2-*luxCDABE* transposon |

Southern blotting was employed. Genomic DNA was digested with SphI. The fragments were electrophoresed through a 1% agarose gel and transferred using a blot apparatus (Stratagene, Heidelberg, Germany) to a positively charged nylon membrane (Hybond-N+; Amersham, Cleveland, OH). The transferred DNA was UV fixed, and the blots were hybridized with a digoxigenin (DIG)-coupled probe. The probe was prepared and detected using DIG High-Prime DNA labeling and detection starter kit I (Roche, Mannheim, Germany) according the manufacturer's instructions. Probes for the detection of the transposons were constructed by PCR amplification of the Tn*5* kanamycin cassette using Kan res-v and Kan res-r primers (17) (Table 2).

The insertion position of the transposon was determined by two different techniques, SphI cloning and arbitrary PCR. DNA sequences flanking transposon insertions were determined by arbitrary PCR as described previously (18), with some modification. Briefly, we performed two rounds of PCR amplification using a degenerate arbitrary primer to anneal to the chromosome and one specific to the mini-Tn*5* transposon. Primers used in the first round were ARB6 and luxCext2 (Table 2). First-round reaction conditions were as follows: (i) 5 min at 95°C; (ii) 6 cycles of 30 s at 95°C, 30 s at 30°C, and 1 min at 72°C; (iii) 30 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C; and (iv) 5 min at 72°C. The second round of PCR amplification used 5 μl purified first-round PCR product as the template and primers ARB2 and luxCint2 (Table 2). Second-round reaction conditions were as follows: (i) 30 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C, and (ii) 5 min at 72°C. The PCR products were purified from an agarose gel and ligated into the vector pCR 2.1-TOPO.

Sequencing reactions were carried out to determine the transposon integration sites using the Seq_O primer (Table 2), which binds to the O end of the mini-Tn*5* transposon. Integration sites were determined using the online BLAST search engine (http://www.ncbi.nlm.nih.gov./).

For the SphI cloning, genomic DNA was digested with SphI. This cleaved the i end of the transposon, including the kanamycin resistance gene. The digested DNA was cloned into pUC19 and transferred into *E. coli* DH5α. Bacteria containing a transposon fragment were selected on LB plates containing kanamycin. The sequence of the inserted DNA was found by sequencing with standard M13 primers, Kan res-v and Kan res-r (Table 2).

**Complementation of the Tn*5* mutants using a *B. cenocepacia* H111 cosmid library.** Chromosomal DNA was extracted from *B. cenocepacia* H111 using a standard protocol (19). The DNA was partially digested with EcoRI and ligated into cosmid pRG930 (20). Packaging and of the cosmid into *E. coli* HB101 cells and titer determination were carried out using the Gigapack III Gold packaging reaction kit from Invitrogen as described in the instruction manual but with the omission of the dilution step of the titer determination reaction. The whole cosmid library was pooled and introduced into each transposon mutant by triparental mating (21). Complemented mutants were selected on ABC minimal plates containing streptomycin and spectinomycin. Cosmids were then extracted using a miniprep kit (Qiagen) and the cosmid ends sequenced with the primers pRG930_seq_rv and pRG930_seq_fw (Table 2) to determine the extent of the genomic insert.

**Pathogenicity screen with *C. elegans*.** Screening was carried out on NG agar plates as described previously (22, 23). H111 mutants were grown at 37°C overnight in LB broth in microtiter dishes (Nunc). Fifty microliters of the respective cultures was spread on NG agar plates in 24-well multiplates (Greiner, Germany). Plates containing bacteria were incubated overnight at 37°C. After a brief incubation at room temperature to allow cooling, plates were inoculated with 20 to 30 synchronized L4 worms of the *C. elegans* Bristol N2 wild-type strain (obtained from the *Caenorhabditis* Genetics Center, University of Minnesota, Minneapolis, MN) (24). The plates were then incubated at 20°C, and live worms were counted after 2 days. In the initial screen approximately 230 24-well plates were tested in a single replicate. All mutants identified in the initial screen were retested in five independent replicates.

***G. mellonella* killing assays.** The *G. mellonella* infection assay was performed as described previously (8). Modifications were made to the protocol as follows. To prevent contamination, the Tn*5* mutants' growth medium was supplemented with kanamycin. For hemolymph extraction, larvae were first sterilized by brief immersion in absolute ethanol. Hemolymph was removed using a syringe with a 22-gauge needle. Approximately 100 μl was obtained per larva. Dilutions were plated for bacterial enumeration. Caterpillars in the final larval stage were purchased from Fischerei Brumann, Zurich, Switzerland.

***D. melanogaster* pricking assays.** *D. melanogaster* Canton-S wild-type flies were used for the pricking procedure. The flies were bred on standard cornmeal sucrose medium and kept at room temperature. The assay was performed as described previously (10). Modifications were made to this protocol as follows. Two- to 9-day-old male and female flies were used in this experiment. For anesthetization, ether was used instead of a $CO_2$ pad. For each trial, duplicate sets of 15 flies were used and the experiment was performed in triplicate. The negative-control flies were inoculated with a 10 mM $MgSO_4$ solution. If more than 5 flies died due to pricking injury, the experiment was repeated. The infected flies were incubated at 26°C.

**AHL quantification.** Strains were grown to an $OD_{600}$ of 3.0. Bacteria were collected by centrifugation at 6,500 rpm for 5 min and the supernatant filter sterilized. One hundred microliters of supernatant was mixed with 100 μl exponential-growth-phase *Pseudomonas putida* F117(pAS-C8-$Gm^r$). This strain is highly specific for $C_8$ homoserine lactone (HSL) detection. Commercial $C_8$ HSLs were used as a standard. The cells were incubated in FluoroNunc Polysorp microtiter plates (Nunc Roskilde) for 6 h in the dark at 30°C. Fluorescence was measured with a Lamda Fluoro 320 Plus reader (Bio-Tek Instruments, Winooski, VT), and the relative fluorescence units (RFU) were determined.

**Determination of siderophore production and extraction of pyochelin.** Siderophore activity of the H111 mutants was tested on CAS agar as described previously (25). CAS plates were inoculated with 5 μl culture at an $OD_{600}$ of 1.0, allowed to dry, and incubated at 37°C for ~48 h.

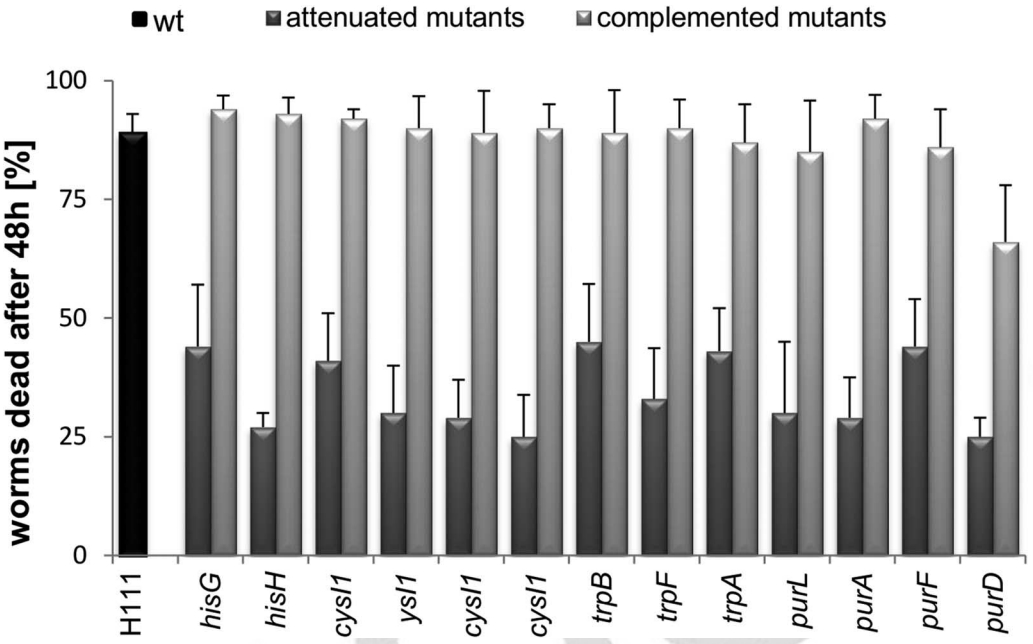


**FIG 1** Virulence of wild-type *B. cenocepacia* H111 and auxotrophic mutants in the *C. elegans* model. *C. elegans* were counted 48 h after spreading the worms on medium covered with a lawn of the respective bacterial strain. Each experiment consisted of three independent replicates. Black bar represents the wild type (wt) (H111), dark gray bars represent mutants grown on NG medium, and light gray bars represent mutants grown on nutritionally supplemented NG medium. Nutritional supplementation was as follows: *hisG* and *hisH*, histidine; *cysI* and *cysB*, cysteine, *trpA*, *trpB*, and *trpF*, DL-tryptophan; *purD*, *purF*, *purL*, and *purA*, adenosine.

Siderophore activity was determined by comparison of halo diameter (a larger halo indicates higher siderophore production) and color (25, 26).

For pyochelin extraction, mutants were grown overnight in 200 ml NG medium to reflect the growth conditions used for the initial identification of mutants attenuated in virulence to *C. elegans*. This ensured both the growth of the mutants and the relevance of the results to pathogenicity in the *C. elegans* model. Bacteria were collected by centrifugation, and the pH of the spent culture supernatants was adjusted to ~2.0 by the addition of HCl. The siderophores were extracted twice by addition of 0.4 volume dichloromethane. The organic phase was removed by rotary evaporation, and the residue was resuspended in 200 to 250 μl methanol.

The extracts were analyzed by thin-layer chromatography on silica 60 plates (VWR) with chloroform-acetic acid-ethanol at 90:5:2.5 (vol/vol) as the solvent (27). Plates were dried after development and siderophores detected by UV light. Pyochelin was identified by the presence of two yellow-green fluorescent bands corresponding to the two pyochelin stereoisomers, pyochelins I and II ($R_f$s, 0.35 and 0.37, respectively), and salicylate was visualized as a blue fluorescent band ($R_f$, 0.74) (28, 29).

**Determination of protease activity and EPS production.** Protease activity was determined on skimmed milk agar as described previously (30). Five microliters of overnight culture from each strain to be tested (diluted to an $OD_{600}$ of 1) was dropped onto the plate and allowed to dry. The plate was incubated at 37°C for 48 h. Extracellular polysaccharides (EPS) production was determined on YEM agar supplemented with Congo red (31). EPS-positive strains showed a slimy, slightly red colony morphology after overnight incubation at 37°C, whereas EPS-negative strains turned red as a result of integration of the dye.

**Production of AidA.** Detection of AidA was performed by Western blotting. Whole-cell proteins were separated on a 15% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Eschborn, Germany). Anti-AidA antibodies (12) were used to probe the membrane. Detection reactions were performed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma, Steinheim, Germany) according to the recommendations of the manufacturer (Roche, Mannheim, Germany).

**Growth on minimal media.** The growth of the transposon mutants was tested using ABC or ABG minimal medium (32). For growth tests, 5 ml of ABC or ABG solution was inoculated with bacteria to an $OD_{600}$ of 0.01 and incubated at 37°C with shaking. For the functional complementation (supplementation) of the purine mutants, 20 mg liter$^{-1}$ adenine, inosine, or guanine was added to ABC minimal medium.

## RESULTS

**Identification of *B. cenocepacia* H111 mutants attenuated in *C. elegans* and their phenotypic characterization.** It has previously been shown that on NG medium the CF isolate *B. cenocepacia* H111 kills *C. elegans* N2 within 3 days. We employed this "slow-killing" assay to screen a collection of approximately 5,500 random mini-Tn*5* insertion mutants of *B. cenocepacia* H111 for attenuated virulence. In total, 23 mutants that reproducibly showed reduced killing after 48 h compared with the wild type were isolated (Fig. 1; Table 3). Previous work has identified several factors that contribute to the pathogenicity of *B. cenocepacia* (7), and we therefore tested the mutants for the production of some of these factors, including siderophores, extracellular polysaccharides (EPS), proteases, AHL quorum-sensing (QS) signaling molecules, and AidA, a protein required for nematode pathogenicity (Table 3).

*B. cenocepacia* H111 produces the two siderophores ornibactin and pyochelin, which were previously shown to be important for virulence in mammals, *C. elegans*, and larvae of the greater wax moth *G. mellonella* but not alfalfa (8, 33, 34). Some of the mutants showed reduced siderophore production (Table 3). In addition, we observed that some of the mutants formed a yellow rather than a pink halo on CAS agar (Fig. 2A), which is indicative of a loss of pyochelin production (35). Extraction of culture supernatant and

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**TABLE 3.** *B. cenocepacia* H111 mutants attenuated in *C. elegans*

| Strain | Gene disrupted | H111 gene ID | Mean (SD) % dead *C. elegans* larvae after 48 h[a] | *D. melanogaster* survival time (h) | Mean (SD) % surviving *G. mellonella* after 48 h | AHL production[a] | Protease activity[a] | EPS production[a] | Siderophore production[a] | Expression of AidA[a] | Growth on ABC[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HIII | None (wt) | | 89 (4) | <72 | 10 (10) | + | + | + | + | + | + |
| A4 | *purD* | 358071918 | 24 (4) | >336 | 72 (18) | < | < | − | − | + | − |
| D9 | *purF* | 358073772 | 44 (10) | >336 | 29 (8) | + | < | < | + | + | − |
| C2-1 | *purL* | 358073038 | 30 (8) | >336 | 50 (20) | < | + | − | − | + | − |
| F8 | *purA* | 358073158 | 29 (8) | <72 | 0 (0) | + | + | < | < | + | − |
| A11 | *aroK* | 358076029 | 25 (4) | <288 | 93 (12) | + | + | − | +[c] | + | − |
| H2 | *ilvC* | 358071882 | 21 (9) | >336 | 27 (25) | + | + | < | − | + | − |
| C5 | *cysI* | 358073512 | 29 (8) | <72 | NT[d] | + | + | < | +[d] | + | − |
| E10 | *cysI* | 358073512 | 41 (10) | <72 | NT | + | + | < | +[d] | + | − |
| F7 | *cysI* | 358073512 | 30 (16) | <72 | NT | + | + | < | +[d] | + | − |
| B1 | *cysI* | 358073512 | 25 (9) | <72 | NT | + | + | < | +[d] | + | − |
| G11 | *cysB* | 358073513 | 62 (8) | <72 | NT | + | + | + | +[d] | + | + |
| E11 | *ahcY* | 358071424 | 11 (9) | <72 | NT | + | + | − | + | + | − |
| E8 | *hisG* | 358075994 | 44 (12) | <72 | NT | + | + | < | + | + | − |
| G5-1 | *hisH* | 358075989 | 27 (3) | <72 | NT | + | + | < | < | + | < |
| D1 | *trpA* | 358073777 | 43 (9) | <72 | NT | + | + | − | + | − | − |
| G5-2 | *trpB* | 358073779 | 45 (12) | <72 | NT | + | + | + | + | < | − |
| B8 | *trpF* | 358073780 | 33 (10) | <72 | NT | < | + | − | + | + | − |
| C2-2 | *gatA* | 358076086 | 63 (15) | <72 | NT | + | < | + | + | + | + |
| E5 | *rsuA* | 358074812 | 29 (9) | <72 | NT | < | − | + | − | < | + |
| G3 | *lon* | 358073031 | 46 (10) | <72 | NT | < | − | + | + | + | + |
| E12-2 | *rsaM* | 358072205 | 17 (8) | <72 | NT | + | < | + | < | < | + |
| D6[b] | *pyrD* | 358074754 | 23 (7) | >336 | 87 (15) | < | − | − | − | + | − |
| G1 | *cepR* | 358072206 | 7 (5) | <72 | 0 (0) | − | − | < | + | + | + |

[a] +, wt level; <, less than wt level; −, absence.
[b] This strain showed a reduced growth rate in LB medium (see Fig. S1 in the supplemental material).
[c] Halo on CAS agar of normal size but pink color absent.
[d] NT, not tested.

analysis by thin-layer chromatography confirmed that these mutants produced no or reduced amounts of pyochelin (Fig. 2B).

The production of extracellular polysaccharides (EPS) is considered to enhance persistence of Bcc species in the lung, similar to alginate in *Pseudomonas aeruginosa* (36, 37). We tested the attenuated mutants for EPS production on YEM agar, which stimulates the production of the major Bcc polysaccharide cepacian (38, 39). Many of the attenuated mutants showed reduced EPS production (Table 3). However, a defined cepacian-deficient mutant of *B. cenocepacia* K56-2 was found to be as pathogenic as the wild type in *C. elegans* and in *G. mellonella* (data not shown), suggesting that the lack of EPS production is not responsible for the reduced virulence of our mutants. It should be noted that cepacian production has been shown to be important for pathogenicity in murine infection models (40, 41). Furthermore, our phenotypic characterization revealed that several of the mutants produced no or reduced amounts of AHL signal molecules, proteases, or AidA (Table 3).

Mutant D6 grew slower in LB medium, whereas the growth rates of the other mutants were indistinguishable from that of the wild type (see Fig. S1 in the supplemental material). In contrast to the wild type, however, many of the mutants showed poor or no growth on minimal medium supplemented with either glucose or citrate as a carbon source and thus were auxotrophic mutants.

**Locations of transposon insertions within the attenuated mutants.** The locations of the transposon insertions were determined by sequencing the DNA regions flanking the transposon (Table 3). To our surprise, only one of the disrupted genes coded for a previously described virulence factor (the QS regulator CepR), while 19 of the mutations were in metabolic genes, three mutations were in regulatory genes, and three mutants were found to have lost an entire replicon (R12, R33, and R40). The latter three mutants have been described elsewhere (42).

Four of the genes identified in the transposon screen (*cepR*, *lon*, *rsuA*, and *rsaM*) code for regulatory proteins. The isolation of a *cepR* mutant is in full agreement with previous work that has demonstrated that the QS regulator CepR is essential for virulence in multiple infection hosts (8). One mutant was found to carry the transposon insertion in the *rsuA* gene. An *rsuA* mutant was previously identified in a screen for biofilm-defective mutants, and this gene (previously known as *yciL*) has been characterized as a higher-level quorum-sensing (QS) regulator (43). The transposon insertion site of another mutant that was affected in QS was mapped to the intergenic region between the divergent *rsaM* and *cepR* genes, such that expression of both genes is affected (data not shown). Finally, the *lon* gene codes for an ATP-dependent protease which belongs to the AAA$^+$ (ATPases associated with a variety of cellular activities) superfamily of enzymes (44). This is a widespread family of enzymes (45), responsible for diverse functions, including protein unfolding, DNA replication, and recombination (44). Disruption of the *lon* gene has been shown to reduce the pathogenicity of *Salmonella enterica* (46), *Pseudomonas syringae* (47), *Campylobacter jejuni* (48), *Agrobacterium tumefaciens* (49), and *Brucella abortus* (50). This appears to be due to the

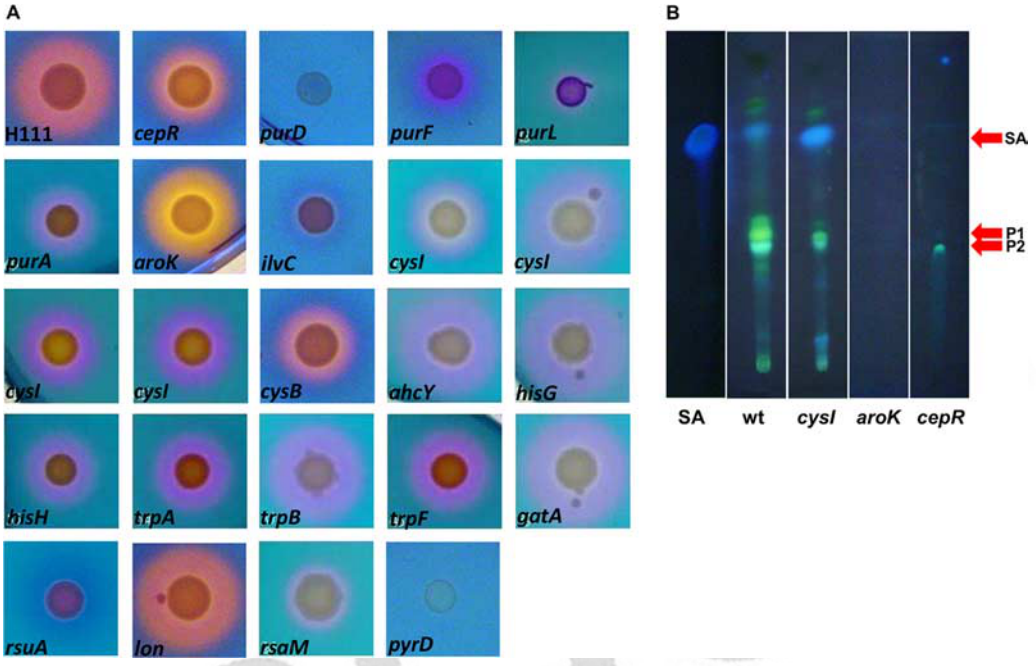


**FIG 2** Siderophore production of attenuated *B. cenocepacia* H111 Tn*5* mutants. (A) Mutants were inoculated on CAS plates and incubated at 37°C for 48 h. The halo diameter corresponds to siderophore activity. A pink-tinted halo is indicative of pyochelin production. (B) Production of pyochelin as determined by thin-layer chromatography. Bands corresponding to pyochelins I and II (P1/P2) and salicylate (SA) are indicated. Extracts were as follows: SA, salicylate standard; wt, wild-type H111; *cysI*, H111 *cysI*; *aroK*, H111 *aroK*; *cepR*, H111 *cepR*.

role of Lon in the upregulation of type three secretion systems (44, 51, 52).

The 19 mutants defective in metabolic functions had mutations that fell within three major pathways: the purine biosynthetic pathway, the shikimate pathway, and the pyrimidine biosynthetic pathway. Four of the attenuated mutants identified during the *C. elegans* screen carried insertions within genes of the purine biosynthesis pathway (*purA*, *purF*, *purL*, and *purD*) (Fig. 3), suggesting that *de novo* purine biosynthesis plays an important role in the nematode pathogenicity of *B. cenocepacia* H111. The *purF*, *purD*, and *purL* genes are all positioned in the initial part of the purine biosynthetic pathway (Fig. 3), and upon inactivation of any of them, one would expect complete abrogation of purine production. The *purA* gene, however, is positioned later in the pathway and would not be necessary for the production of guanine- and xanthine-related purines. IMP is the first molecule containing the purine double-ring system to be formed in the purine pathway. This molecule can be used in the production of both adenine and guanine by the cell (53, 54) (Fig. 3). Growth of the *purD*, *purF*, and *purL* mutants could be successfully restored by the addition of inosine to ABC minimal medium (Fig. 4C, D, and E). Supplementation with adenine, but not with guanine, re-

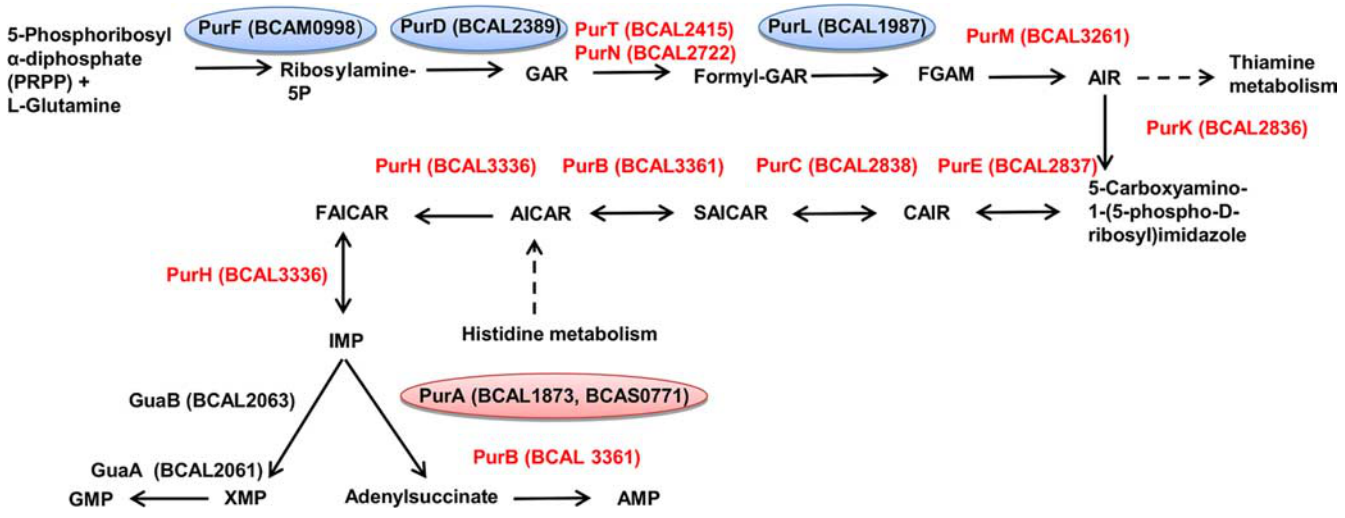


**FIG 3** The *B. cenocepacia* purine biosynthetic pathway. Mutants with attenuated virulence isolated in this study are circled.

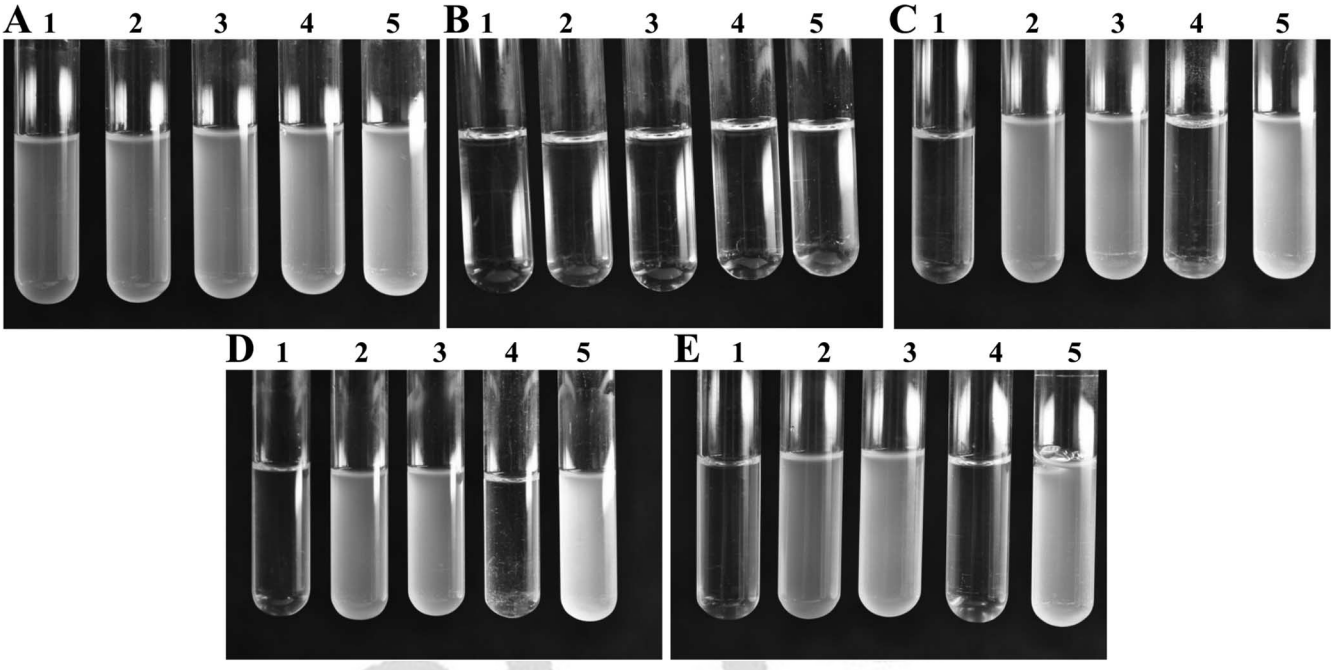


**FIG 4** Growth of purine mutants in ABC medium supplemented with various metabolites. Tubes: 1, unsupplemented medium; 2, supplementation with inosine; 3, supplementation with adenine; 4, supplementation with guanine; 5, supplementation with adenine and guanine. (A) *B. cenocepacia* HIII; (B) *purA* mutant; (C) *purD* mutant; (D) *purF* mutant; (E) *purL* mutant.

stored growth to these mutants in ABC minimal medium (Fig. 4C, D, and E). Adenine can be converted to guanine via the purine metabolic pathway, but the reciprocal conversion is not possible via this pathway. The addition of inosine, adenine, and guanine to ABC medium did not affect the growth of wild-type H111 (Fig. 4A).

The *pyrD* and *aroK* mutants showed very similar phenotypic characteristics (Table 3). The *pyrD* gene codes for a class 2 dihydroorotate dehydrogenase, which is a key component in pyrimidine metabolism. The *aroK* gene encodes a component of the shikimate pathway, through which chorismate, an important precursor in the production of aromatic amino acids, is produced. Another mutant was disrupted in *ilvC*, a ketol-acid reductoisomerase, which plays a role in valine, leucine, and coenzyme A biosynthesis.

Of the remaining metabolic mutants, 10 had defects in amino acid biosynthesis. Four cysteine pathway mutants were all disrupted in the *cysI* gene (which codes for the beta subunit of a sulfide reductase), and one mutant bore an insertion in the *cysB* gene (a potential transcriptional regulator of the *cys* regulon). Two additional genes that play a role in the histidine metabolic pathway, *hisG* (encoding an ATP-phosphoribosyltransferase), and *hisH* (encoding a glutamine-amidotransferase), were found to be important for pathogenicity in the *C. elegans* model, as were the tryptophan biosynthetic pathway genes *trpA*, *trpB*, and *trpF* [encoding tryptophan-synthase alpha and beta chains and *N*-(5′-phosphoribosyl)anthranilate-isomerase, respectively].

The final two metabolic mutants bore insertions in genes not directly involved in amino acid biosynthesis; the *ahcY* (*S*-adenosylhomocysteine hydrolase) gene, the product of which acts as a coenzyme in cysteine and methionine metabolism, and the *gatA* (glutamyl-tRNA amidotransferase subunit A) gene, which plays a role in protein synthesis.

**Five of the mutants attenuated in *C. elegans* are also less virulent in *D. melanogaster* and *G. mellonella*.** Previous work has shown that some virulence factors are host specific, while other factors are important for pathogenicity in multiple infection models (8). We tested the 23 mutants showing attenuation in the *C. elegans* pathogenicity assay using the *D. melanogaster* infection model to discern host-specific and general factors. The fruit fly *D. melanogaster* has been shown to be a useful nonmammalian infection host for the determination of the pathogenicities of different Bcc strains (9). Of the 23 mutants with reduced *C. elegans* virulence, six were also strongly attenuated in the *D. melanogaster* infection model (Fig. 5). These six mutants were further tested for virulence using larvae of the greater wax moth *G. mellonella* as a host in order to validate their function as general virulence factors (Fig. 6). Five of the six mutants were attenuated compared to the wild type in this model, namely, the *purD*, *purF*, and *purL* purine biosynthesis mutants, the *pyrD* pyrimidine biosynthesis mutant, and the *aroK* mutant, which is defective in aromatic amino acid biosynthesis. The *ilvC* mutant showed attenuation in *C. elegans* and *D. melanogaster* but not in *G. mellonella*. It is therefore tempting to speculate that in *G. mellonella* but not in the other infection hosts tested, sufficient amounts of certain metabolites are available to rescue the defects of the *ilvC* mutant. In contrast to the *purD*, *purF*, and *purL* mutants, the *purA* mutant exhibited wild-type pathogenicity in both the *D. melanogaster* and the *G. mellonella* infection models (Table 3). *In silico* analysis of the *B. cenocepacia* H111 genome revealed a homologous gene product (GeneID 358069869) with 51.5% identity to H111 PurA. It is possible that this gene is functionally similar to *purA* and is able to at least partially rescue the production of adenine-related purines in *D. melanogaster* and *G. mellonella*. The quorum-sensing regulator CepR was previously shown to be important for pathogenicity to *C. elegans* but not to *G. mellonella* (8), and our results show that

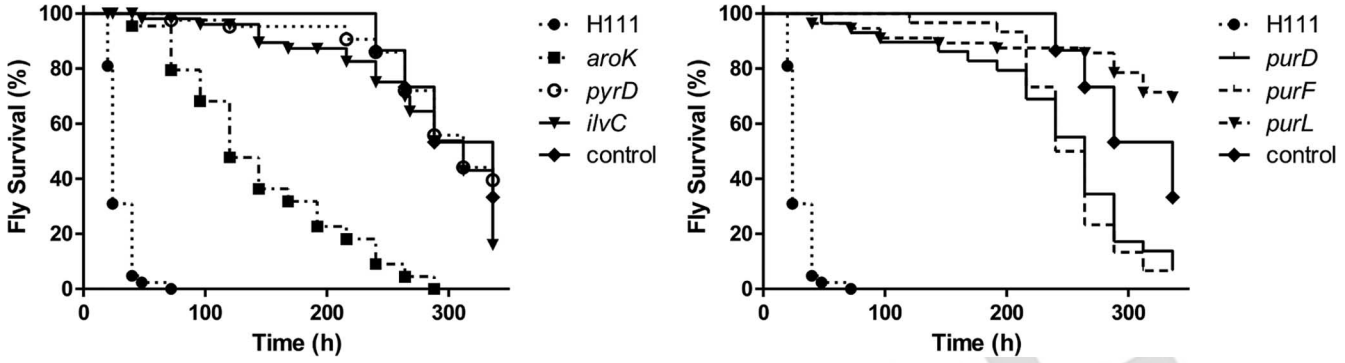


**FIG 5** Virulence of wild-type *B. cenocepacia* H111 and attenuated auxotrophic Tn*5* mutants in the *D. melanogaster* infection model. Fifteen flies were inoculated with bacterial culture per experiment and incubated at 26°C. Live flies were counted every 24 h postinfection. Data are based on three independent experiments and were analyzed using survival curves generated by the Kaplan-Meier statistical method. The significance of the difference between results for the wild type and the mutants was determined using the log rank (Mantel-Cox) test. For all the tested strains the *P* value was $<0.0001$.

this regulator is also not essential for pathogenicity to *D. melanogaster* (Table 3).

In summary, five mutants were identified which were attenuated in all three infection hosts used. The genes inactivated in these strains (*aroK*, *pyrD*, *purD*, *purF*, and *purL*) may therefore encode factors that are universally important for virulence, whereas the genes inactivated in the other mutants were specific virulence factors for *C. elegans* and/or *D. melanogaster*.

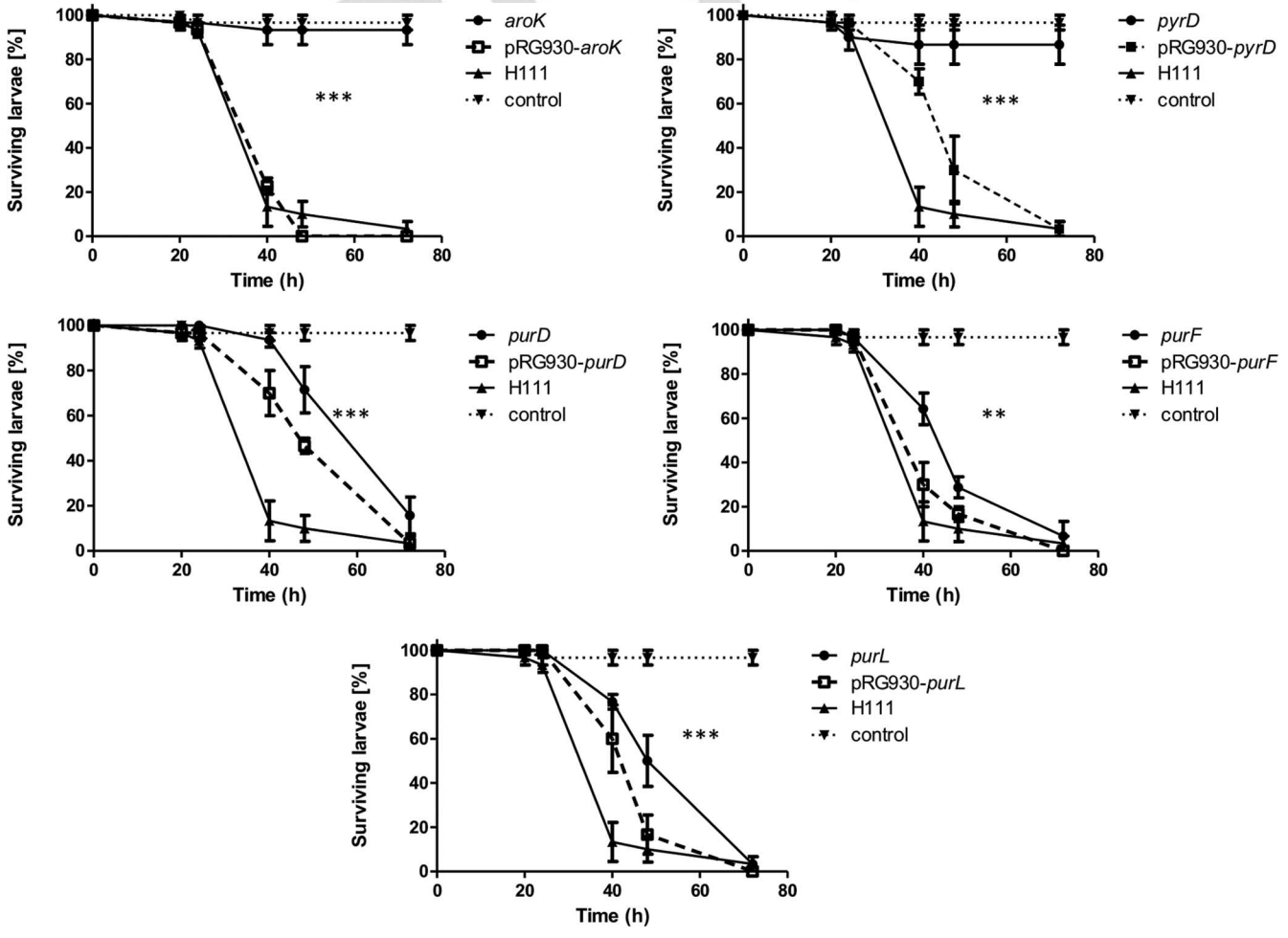


**FIG 6** Virulence of wild-type *B. cenocepacia* H111type, attenuated auxotrophic Tn*5* mutants, and complemented mutant strains in the *G. mellonella* infection model. *G. mellonella* larvae were infected with approximately $2 \times 10^5$ bacteria and incubated at 30°C in the dark. Live and dead larvae were counted after 20, 24, 40, 48, and 72 h postinfection. The curves were calculated using three independent replicates. The significance of the difference between results for the wild type and the Tn*5* mutants was determined using the log rank (Mantel-Cox) test and is indicated as follows: *, $0.01 \leq P \leq 0.05$; **, $0.001 \leq P \leq 0.01$; ***, $P < 0.001$.

**Complementation and supplementation of the Tn*5* metabolic mutants.** The *aroK*, *pyrD*, *purD*, *purF*, and *purL* mutants, which showed attenuation in all the animal models tested, were complemented using a *B. cenocepacia* H111 cosmid library. All complemented mutants were able to grow at the wild-type rate on minimal medium with citrate as a carbon source (see Fig. S2 in the supplemental material). Furthermore, pathogenicity of the complemented mutants was at least partially restored (Fig. 6). Supplementation of nematode growth medium with histidine, cysteine, or adenosine as appropriate restored virulence to wild-type levels in the *C. elegans* model in all metabolic mutants except the *purD* mutant, which showed an intermediate level of virulence (Fig. 1; Table 3).

## DISCUSSION

Over the past few years, nonmammalian infection models have been established as attractive alternatives to traditional animal models because of their practical advantages, particularly the possibility of performing high-throughput screens. In this study, we used the nematode *C. elegans* as an infection host to screen a *B. cenocepacia* H111 mutant library for attenuated strains.

Previous work has shown that the CepIR QS system is crucial for *B. cenocepacia* virulence in *C. elegans*, as it controls the expression of factors that contribute to pathogenicity in this infection model (8, 22). The finding that in one of the attenuated mutants the transposon had inactivated *cepR* and in two other mutants (the *rsuA* and *lon* mutants) it had affected the QS circuitry therefore validates the screening strategy. One of the QS-regulated virulence factors that has been shown to be important for killing of *C. elegans* is the protein AidA, although its mode of action remains to be elucidated (12). Interestingly, AidA has not been found to play a role in any other infection host tested so far, and it thus appears to be a specific virulence factor required for infection of nematodes (8). The finding that the *trpA* and *trpB* mutants produce no or greatly reduced amounts of AidA may contribute to the specific attenuation of these strains in the *C. elegans* model (Table 3). Supplementing the medium with tryptophan restored AidA production as well as virulence of the two mutants (Fig. 1 and data not shown), indicating that the attenuation of the two mutants is not a consequence of a secondary mutation. However, at present it is unclear how *trpA* and *trpB*, which are required for tryptophan biosynthesis, affect expression of AidA.

Other QS-regulated virulence factors of *B. cenocepacia* include the ZmpA and ZmpB proteases and the siderophore pyochelin (8). Proteolytic activity was shown to be important for pathogenicity in mammals but not in invertebrates or alfalfa, likely because they specifically modulate the host immune response of mammals by degrading specific tissue components such as collagen and fibronectin and by obstructing immune proteins (55–57). Hence, the abolished or lowered proteolytic activity observed for several of the mutants (Table 3) does not account for their lowered virulence in the infection models used. However, one would expect that these mutants would be attenuated in mammals. Pyochelin production in *B. cenocepacia* has been shown to be dependent on the availability of its precursors, salicylic acid and cysteine (58). As expected, all five mutants with insertions in the genes of the cysteine biosynthetic pathway showed a reduction in pyochelin production (Fig. 2) and an increase in the accumulation of its precursor salicylic acid. Likewise, the *aroK* mutant, which is defective in the biosynthesis of aromatic metabolites, including the pyochelin precursor salicylic acid, did not produce pyochelin.

Our mutant screen identified a few genes that were essential for virulence in all three nonmammalian infection models used in this study. Rather than coding for typical virulence factors, these genes were *purF*, *purD*, and *purL* from the purine biosynthesis pathway, *pyrD* from the pyrimidine synthesis pathway, and *aroK* from the shikimate pathway. Given that *B. cenocepacia* produces a battery of virulence factors (7), the inactivation of just one of these may not greatly affect the overall pathogenicity of the organism, explaining why such factors were not identified in our screen. The genes identified encode enzymes that are critical for essential anabolic pathways, and consequently these mutants were unable to grow in minimal medium. Importantly, auxotrophy *per se* is not the reason for the reduced virulence of these strains, as we determined that approximately 8% of the mutants in the transposon insertion library were unable to grow in minimal medium. This suggests that it is the lack or shortage of specific metabolites in each infection host that causes attenuation. In agreement with this hypothesis, we observed that nematode pathogenicity of the mutants could be restored to wild-type levels by supplementing the medium with appropriate metabolites (Fig. 1). It is important to note, however, that on NG medium, which was used for the *C. elegans* assays, the mutants showed no growth defects, suggesting that the reduced virulence is not a just a consequence of a lowered infection dose. In the case of the *aroK* mutant, we tested whether the strain could persist within infected *G. mellonella* larvae. At 8 days postinfection the animals did not show any disease symptoms, yet we were able to isolate the mutant from the hemolymph of the larvae (approximately 550 bacteria per μl), demonstrating that the bacteria were capable of *in vivo* survival. We hypothesize that the nutritional environment in the infection host supports persistence of the mutant but neither significant growth nor energy-consuming virulence factor production.

Although it remains to be determined whether our *B. cenocepacia* mutants are also attenuated in a mammalian infection host, it is notable that the same metabolic pathways have been identified as key systems in virulence in murine models. *De novo* purine biosynthesis has been shown to be essential for the virulence of a variety of pathogens, including *Francisella tularensis* (59), *Salmonella enterica* serovar Typhimurium (60), *Staphylococcus aureus* (61), *Streptococcus pneumoniae* (62), *Yersinia pestis* (63), *Vibrio vulnificus* (64), *Bacillus anthracis* (54, 65), *Brucella melitensis* (66), and *Brucella abortus* (67). Previous work has also shown that in *V. vulnificus*, pyrimidine biosynthetic genes are preferentially expressed during infection, and a *pyrH* mutant was attenuated in virulence (64, 68). In *Listeria monocytogenes*, both purine and pyrimidine biosynthetic genes were found to be upregulated in infected mammalian cells (69). In a recent study, it was demonstrated that *de novo* nucleotide biosynthesis is critical for survival and growth of bacteria in human serum, and therefore the purine and pyrimidine biosynthetic pathways are essential for proliferation of bacterial pathogens in the bloodstream (70). Finally, the shikimate pathway, of which the AroK protein is a part, has been demonstrated to be required for virulence of *S. enterica* serovar Typhimurium (71), *P. aeruginosa* (72), and *L. monocytogenes* (73). Most interestingly in the context of this study is the finding that inactivation of *aroB*, which is required for shikimate biosynthesis in *Burkholderia pseudomallei*, the causative agent of melioidosis, renders the organism avirulent. Moreover, murine challenge

studies revealed partial protection in BALB/c mice vaccinated with an *aroB* mutant (74). Given that the shikimate pathway is crucial to bacteria but missing in mammals (75), the enzymes involved in this pathway have been considered particularly interesting drug targets for developing nontoxic antimicrobial agents (76).

In summary, we have identified several genes in *B. cenocepacia* that are critical for pathogenicity in multiple nonmammalian infection hosts. These results not only further our understanding of the virulence mechanisms used by this opportunistic pathogen but also have led to the identification of some potential targets for the development of novel antibacterial drugs. Some of the strongly attenuated mutants identified in this study could also be of interest for the development of live vaccines.

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## 3.4 Chromosome three of Bcc strains is essential for virulence in *C. elegans* and *G. mellonella*

The strong attenuation of three *B. cenocepacia* H111 transposon mutants (R12, R33 and R40) in *C. elegans, G, mellonella* and alfalfa together with the fact that defined knockout mutants in the identified genes showed no effect in pathogenicity led us to investigate these mutants in better detail. Phenotypic and genotypic characterisation revealed, that chromosome three, which so far was considered to be indispensable, is missing in these three mutants. However, it was unclear if the loss of pathogenicity was a consequence of the loss of the chromosome three (which was designated plasmid pC3) or if other spontaneous mutations have occurred in these three mutants. In 1994 Cheng and Lessie showed that chromosomes can undergo rearrangements, but they did not observe the loss of entire replicons (Cheng & Lessie, 1994). Kirsty Agnoli from our group has developed a method using plasmid incompatibility to eliminate chromosome three of Bcc species namely *B. ambifaria, B. anthina, B. cenocepacia, B. ubonensis* and *B. vietnamiensis* (Agnoli *et al.*, 2012). Like R12, R33 and R40 these mutants showed no defect in growth and only a few changes in metabolic functions were observed (Agnoli *et al.*, 2012). Investigations of the pathogenicity of the ΔpC3 strains confirmed results from the three Tn*5*-insertion mutants. All the wild-type strains, which showed a very high pathogenicity in the *C. elegans* model, were totally attenuated when chromosome three was eliminated. *B. cenocepacia* H111, *B. cenocepacia* H12424, *B. lata* 383 and *B. ubonensis,* LMG20358 were the wild-type stains, which were most pathogenic. The ΔpC3 derivatives of these strains were totally attenuated with a score of zero. For *B. anthina* LMG20983 the pathogenicity score decreased from a score of two (wild-type) to zero. For all the other strains tested (*B. ambifaria* LMG19182, *B. cenocepacia* MCO-3, *B. pyrrocinia* LMG14191 and *B. vietnamiensis* LMG10929) no difference could be detected because the wild-type strains showed no virulence. In the *G. mellonella* infection model the tested ΔpC3 strains, *B. cenocepacia* H111, *B. cenocepacia* H12424, *B. pyrrocinia* LMG14191, *B. ubonensis* LMG20358 and *B. ambifaria* AMMD showed to be attenuated compared to the wild-type. No effect was observed for *B. anthina* LMG20983 because the wild-type showed only very little pathogenicity when tested in *G. mellonella*. For *B. cenocepacia* MCO-3, *B. vietnamiensis* LMG10929 and *B. lata* 383 the Wilcoxon test showed no significant differences between the wild-type and the ΔpC3 mutants. The importance of pC3 in virulence was also shown in experiments using rats and zebra fish as infection hosts. The results from these experiments showed that this replicon codes for genes essential for pathogenicity for Bcc strains. Because the gene *aidA* which encodes a protein required for nematode killing, the *C. elegans* model can be explained by the lack of this gene ΔpC3 derivatives (Huber *et al.*,

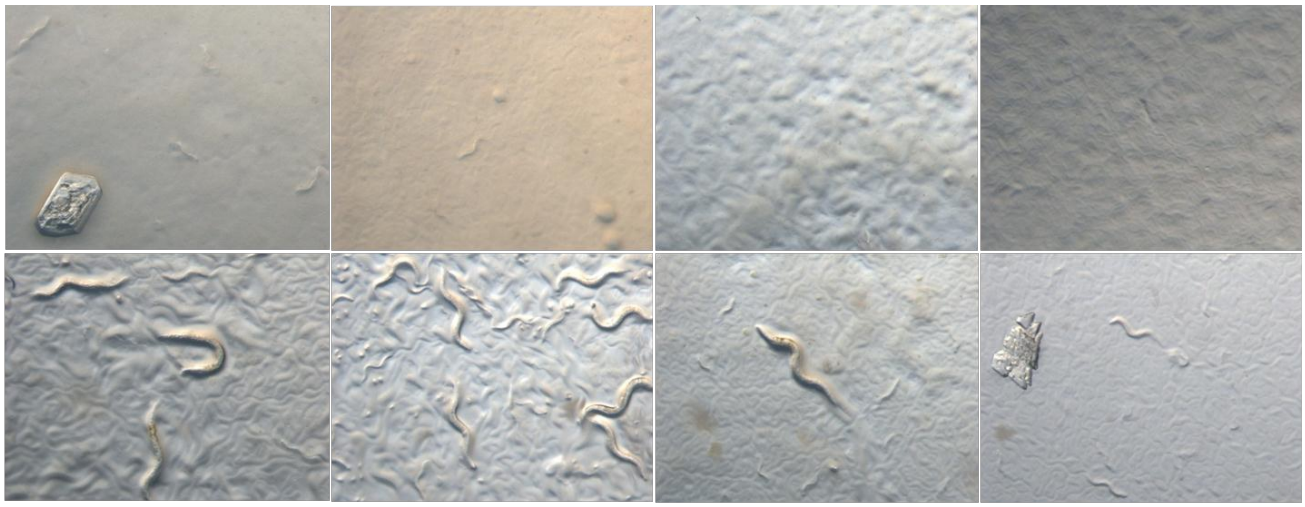
2004). No correlation between the biosynthesis of AidA and the pathogenicity in *G. mellonella* could be observed when different Bcc strains were tested (data not shown). This suggest that additional virulence genes are located on chromosome three which are required for the killing of *G. mellonella*. However, additional work is required to elucidate their identity.

### 3.4.1 A conserved role of pC3 in pathogenicity of Bcc

Except for *B. cepacia,* from which pC3 could so far not be eliminated, ΔpC3 derivatives from all remaining Bcc were constructed. In addition to the nine strains described in the chapter above ΔpC3 derivatives of *B. diffusa* LMG24065, *B. multivorans* LMG18825, *B. metallica* LMG20468, *B. contaminans* LMG23361, *B. seminalis* LMG24067, *B. latens* LMG24064, *B. arboris* LMG24066 and *B. stabilis* LMG7000 were constructed. The ΔpC3 strains were tested in the *C. elegans* feeding model and in agreement with the results of the nine Bcc species described above all new ΔpC3 strains showed attenuation when compared to the wild type strains (Figure 6). Noteworthy, *B. multivorans, B. latens* and *B. arboris* were not pathogenic in the *C. elegans* model (Table 3). By introduction of an *oriT* into pC3 of *Burkholderia cenocepacia* H111 it was possible to transfer this replicon to pC3-null versions of other Bcc species. This resulted in an increase in pathogenicity to approximately the level of the pC3 donor strain (Table 3, Figure 5). To localise genes which play a role in pathogenicity, the sequence of pC3 from *B. cenocepacia* H111 was compared with those from other completed Bcc genomes. The leading half of pC3 was found to be conserved, while the remainder showed little conservation between species. This led us to construct a partial deletion of the non-conserved part of *B. cenocepacia* H111 pC3 by FLP/FRT recombination. This partial deletion did not affect pathogenicity in either *C. elegans* or *G. mellonella* (Table 3, Figure 5 and 7), suggesting that the pathogenicity genes of pC3 are located on the conserved part of the replicon. Furthermore, we used a broad-host-range cosmid library to conduct a gain-of-function screen in *B. cenocepacia* H111Δc3. Of 2,000 mutants tested, only one was found to restore pathogenicity in *C. elegans* (Table 3). Tests showed that this cosmid did not carry the *aidA* gene. Further work will be required to identify the genes on the cosmid that confer virulence.

***E. coli* OP50** ***B. cenocepacia* H111** ***B. cenocepacia* H111 ΔpC3** ***B. cenocepacia* H111 ΔpC3+KC3**

**Figure 5:** *C. elegans* feeding assays 120 h post inoculation. Worms were grown on NGMII plates seeded with different Bcc strains. From left to rights *E. coli* OP50 (food source strain), *B. cenocepacia* H111 wild type, *B. cenocepacia* H111ΔpC3 and *B. cenocepacia* H111ΔpC3/pC3$_{K56-2}$. The pictures were taken with a Leica Binocular M165FC using a 50x magnification.



***B. contaminans* LMG23361** ***B. diffusa* LMG24065** ***B. metallica* LMG24068** ***B. seminalis* LMG24064**

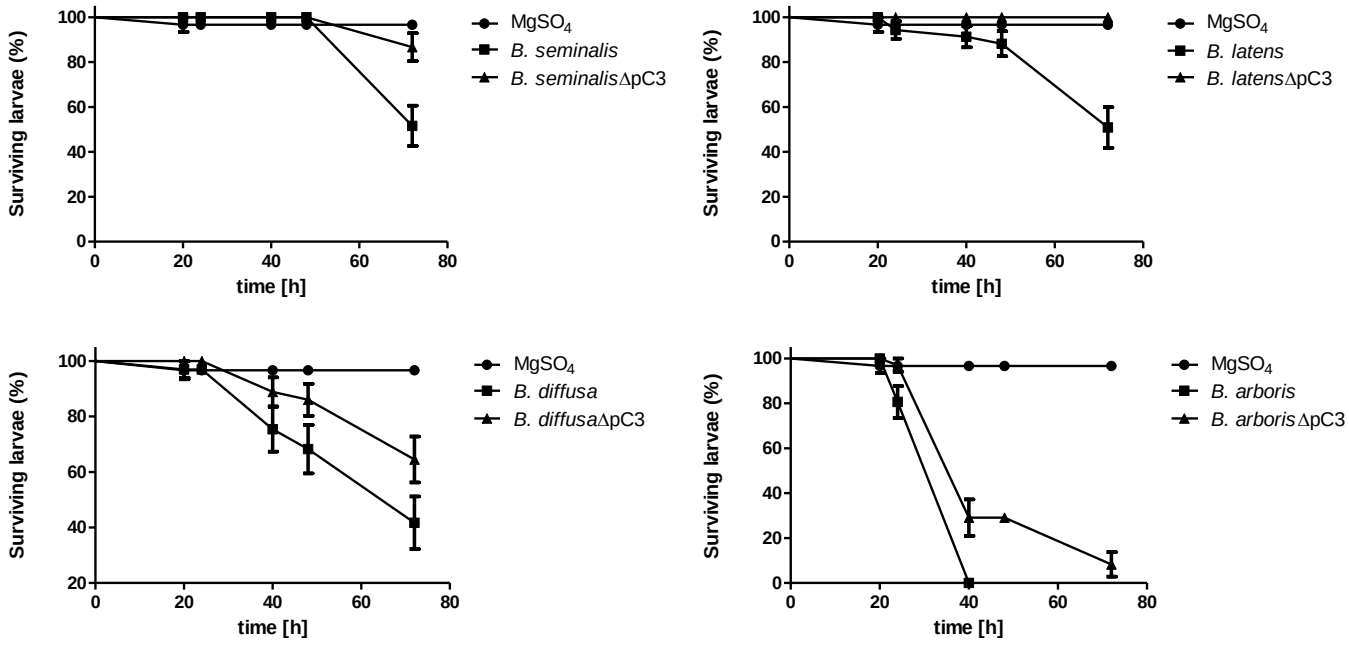
**Figure 6:** *C. elegans* feeding assays 120 h post inoculation. Worms were grown on NGMII plates seeded with different Bcc strains. The upper panel shows wild type strains and the lower panel the respective ΔpC3 derivatives. The pictures were taken with a Leica Binocular M165FC using a 50x magnification.

| Species | Strain | Appearance (day 2)[b] | % live worms (day 2)[c] | Worms (day 5)[d] | Pathogenicity score[a] |
|---|---|---|---|---|---|
| ***B. cenocepacia*** | H111 | Sick | 56 | 1 | 3 |
| ***B. cenocepacia*** | H111ΔpC3 | Normal | 82 | 100-500 | 0 |
| ***B. cenocepacia*** | H111pC3del1 | Sick | 29 | 3 | 3 |
| ***B. cenocepacia*** | H111ΔpC3/pRG930 | Sick | 54 | 3 | 3 |
| ***B. cenocepacia*** | K56-2 | Sick | 23 | 0 | 3 |
| ***B. cenocepacia*** | H111ΔpC3/pC3$_{K56-2}$ | Sick | 30 | 13 | 3 |
| ***B. diffusa*** | LMG24065 | Sick | 35 | 50-100 | 2 |
| ***B. diffusa*** | LMG24065ΔpC3 | Normal | 80 | 100-500 | 0 |
| ***B. multivorans*** | LMG18825 | Normal | 82 | >500 | 0 |
| ***B. multivorans*** | LMG18825ΔpC3 | Normal | 79 | >500 | 0 |
| ***B. metallica*** | LMG24068 | Sick | 30 | 0 | 3 |
| ***B. metallica*** | LMG24068ΔpC3 | Normal | 76 | 100-500 | 0 |
| ***B. contaminans*** | LMG23361 | Sick | 64 | 100-500 | 2 |
| ***B. contaminans*** | LMG23361ΔpC3 | Normal | 65 | 100-500 | 1 |
| ***B. seminalis*** | LMG24067 | Sick | 32 | 3 | 3 |
| ***B. seminalis*** | LMG24067ΔpC3 | Normal | 73 | 100-500 | 0 |
| ***B. latens*** | LMG24064 | Normal | 87 | >500 | 0 |
| ***B. latens*** | LMG24064ΔpC3 | Normal | 81 | >500 | 0 |
| ***B. arboris*** | LMG24066 | Normal | 86 | 100-500 | 0 |
| ***B. arboris*** | LMG24066ΔpC3 | Normal | 83 | 100-500 | 0 |
| ***B. stabilis*** | LMG7000 | Sick | 56 | 5 | 3 |
| ***B. stabilis*** | LMG7000ΔpC3 | Normal | 95 | 100-500 | 0 |

**Table 3:** Pathogenicity of wild- type Bcc strains and their pC3 deletion derivatives in *C. elegans.* The pathogenicity score [a] was calculated by the visible unhealthiness (swollen intestine, small size, locomotive capacity) at day two [b], percentage of living worms at das two (≤ 70%)[c] and the total number of living nematodes inclusive progenies at day five (≤ 50)[d]. For every criterium met one point was given. A total score of three indicates a very pathogenic strain, where as a final score of zero is indicative of a non-pathogenic strain (Cardona *et al*., 2005). Each experiment was carried out in triplicate.

*B. seminalis, B. latens, B. diffusa* and *B. arboris* ΔpC3 were all less pathogenic as expected when testes in the *G. mellonella* infection model. The *B. contaminans* and *B. metallica* wild type strains were the most pathogenic strains from the Bcc. I have therefore used a lower inoculum size (~ 200'000 bacteria instead of 350'000 – 400'000). However, even under these conditions almost 100 % of the larvae were killed after only 40 h and there was no significant difference between the wild type and the ΔpC3 strains. It would be worth to test even lower infection dosages. For *B. multivorans* and *B. stabilis* a higher infection dosage (1'400'000 to 1'600'000 bacteria in 10 µl inoculum) was used, because first tests using 350'000 – 400'000 cells showed that these strains exhibited only very little pathogenicity, and therefore no

significant difference between the wild type and the ΔpC3 mutant was observed. Even when using four times more bacteria only 30 % of the larvae died after 72 h when *B. multivorans* was used. In the case of *B. stabilis* the larvae died faster with this high concentration but the ΔpC3 derivative killed the larvae as fast as the wild type. To make sure that this is not due to the bad quality of *G. mellonella* larvae during the winter months, this strain should be retested. The *B. cenocepacia* H111ΔpC3/pC3$_{K56-2}$ strain was as pathogenic as the K56-2 wild type. This indicates that the virulence of ΔpC3 could be restored with a pC3 plasmid from a different Bcc strain. No difference in pathogenicity could be observed when the non-conserved region of the pC3 was deleted. This indicates that the genes which are responsible for *G. mellonella* pathogenicity on pC3 are located on the conserved region.



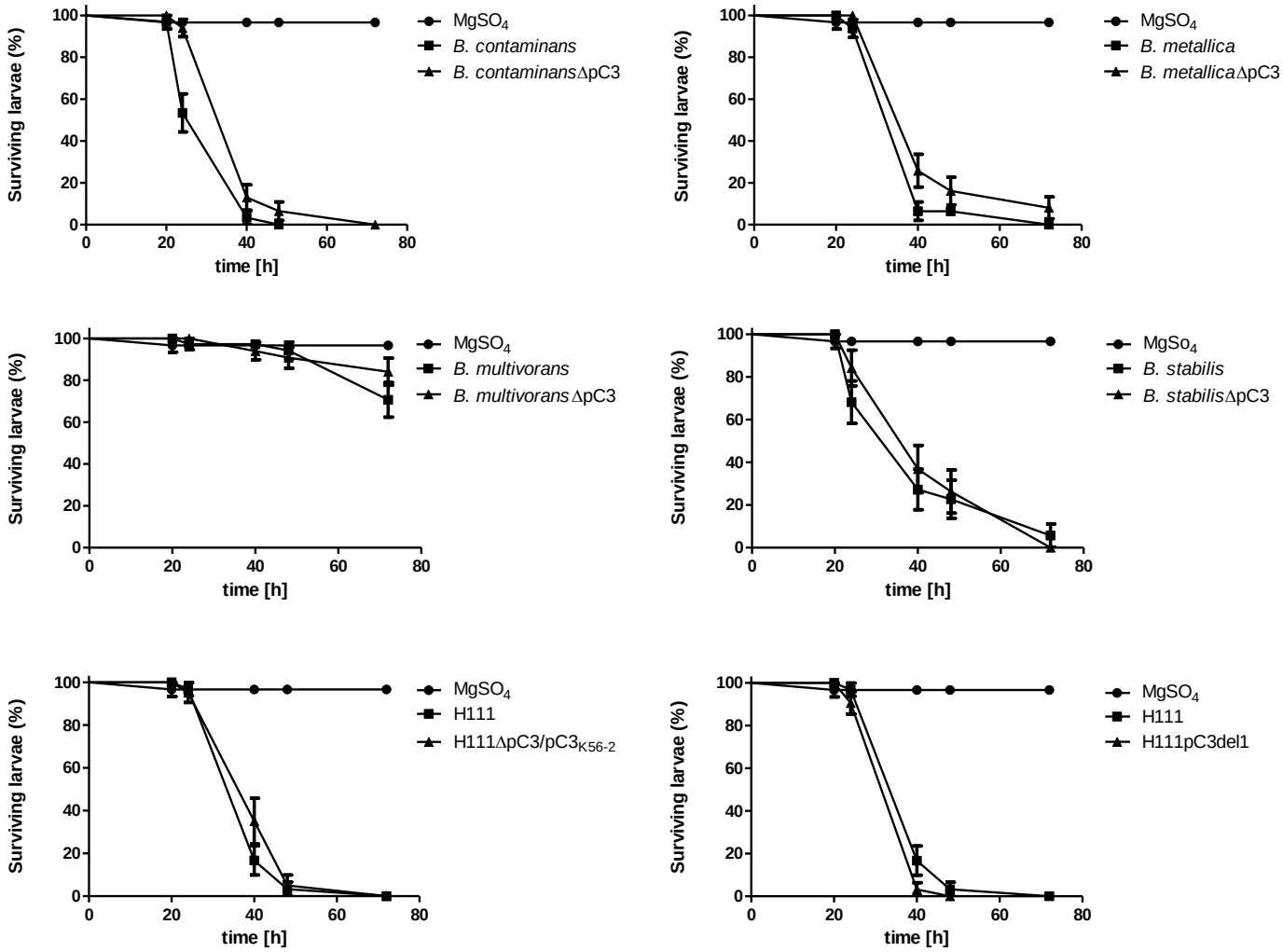



**Figure 7:** *G. mellonella* pathogenicity assay with different strains of the Bcc and their ΔpC3 mutants. 10 µl of the particular strains were injected and the death and alive larvae were counted after 20, 24, 40, 48 and 72 post infection. For *B. seminalis, B. lantens, B. diffusa, B. arboris* and *B. cenocepacia* H111 the bacterial suspension was diluted to around 350'000 – 400'000 bacteria (corresponds to 10 µl culture with an $OD_{600}$ of 0.125), for *B. metallica* and *B. contaminans* with around 200'000 bacteria (corresponds to 10 µl culture with an $OD_{600}$ of 0.0625) in the inoculum and a higher concentration for the less pathogenic strain *B. multivorans* and *B. stabilis* to 1'400'000 – 1'600'000 bacteria (corresponds to 10 µl culture with an $OD_{600}$ of 0.5). For each experiment 10 larvae were used and repeated at least three times. The incubation temperature used for the experiment was 30°C.

## 3.5 LasI/R and RhlI/R quorum sensing system of *P. aeruginosa* PUPa3

*P. aeruginosa* is a Gram-negative bacterium that can colonize many environmental niches. Like other Gram-negative and Gram-positive bacteria the regulation of different genes in *P. aeruginosa* involves cell-cell communication *via* the production of small molecules. The concentrations of this small molecules increases with the bacterial cell density. The result of reaching a critical concentration changes the expression of different target genes. Many virulence factors like swarming, swimming, sporulation, biofilm formation, bioluminescence, protease production, the biosynthesis of antibiotic are regulated by the QS system. The QS system helps the bacteria to adapt rapidly to new environmental niches and conditions. The plant growth-promoting rice rizosphere isolate *P. aeruginosa* PUPa3, which shows antifungal and other plant beneficial activities, was used to study the role of QS in virulence. The *P. aeruginosa* PUPa3 QS system is highly homologous to the system of *P. aeruginosa* PAO1: it consists of two QS systems, the LasI/R and the RhlI/R system, which are almost identical to the LasI/R and the RhlI/R system of the clinical isolate *P. aeruginosa* PAO1. The *lasI* is responsible for the synthesis of *N*-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL) which binds to the response regulator LasR. The *rhlI* synthesis the N-(butanoyl)-homoserine lactone (C4-HSL) which binds to its response regulator RhlR. For *P. aeruginosa* PAO1 it is known that these two systems are organized hierarchically such that the LasI/R system regulates the transcription of *rhlI-rhlR* (Latifi *et al.,* 1996). The results for the pathogenicity of *P. aeruginosa* PUPa3 showed that the environmental isolate *P. aeruginosa* PUPa3 is highly virulent in the tested insect models and only the *lasI rhlI* double mutant showed attenuation in virulence. These results were further supported by phenotypic assays, including swarming, swimming and the rhizosphere colonisation. While in *P. aeruginosa* PAO1 the QS systems are hierarchically organized, the systems in *P. aeruginosa* PUPa3 seem to operate in parallel in spite of their high degree of similarity (Steindler *et al.*, 2009).

## 3.6 Cystic fibrosis-niche adaptation of *P. aeruginosa* reduces virulence in multiple infection hosts

*P. aeruginosa* is an opportunistic pathogen which has broad capabilities to adapt to diverse ecological niches and to establish human infections. I participated in a study to analyse persistence of three *P. aeruginosa* clonal linages in three different non-mammalian hosts. The results were compared with data from a murine infection model. For this study early *P. aeruginosa* AA2, KK1, KK2 and MF1 strains were compared for their virulence against the later isolates AA43, AA44, KK71, KK72 and MF51. The non-mammalian assays used in this study were the *C. elegans* slow-killing, the *D. melanogaster* feeding and pricking and the *G. mellonella* infection models. The results from all the different hosts indicated that there was a clear adaption to the host. The pathogenicity of all the tested strains was decreased the later after infection they were isolated from the cystic fibrosis patients. No host specificity or differences between an acute (*D. melanogaster* pricking, *G. mellonella*) or chronic infection (*D. melanogaster* and *C. elegans feeding*) could be observed (Lore *et al.*, 2012).

# 4 Future perspectives

## 4.1 The genes *purD, purF, purL, aroK* and *pyrD* as potential drug targets

There are still a lot of open questions about the role of specific virulence factors in pathogenesis of *Burkholderia* and *Pseudomonas*. One aim of this study was to identify novel virulence factors and genes, which could serve as potential drug target candidates, by the aid of various non–mammalian animal models. A Tn*5* transposon insertion library was screened and several auxotrophic mutants were isolated, which showed attenuation in all the animal models used. Among these mutants three were from the purine synthetic pathway (*purD, purF* and *purL*). An interesting line of future research will be to investigate the role of genes further downstream in the biosynthesis pathway. Specifically, it would be interesting to analyse the role of *guaB* encoding an IMP (inosine monophosphate) dehydrogenase; *guaA* encoding a GMP (guanine monophosphate) synthetase, in pathogenicity. Until now I was not able to create these mutants even by adding 20 mg/litre guanine to the medium, a condition previously shown to allow the isolation of such mutants (Jewett *et al.*, 2009). Maybe *B. cenocepacia* H111 is not able to take the guanine up from the medium. It would be worthwhile to repeat the experiment with a different technique, namely by constructing a conditional mutant, in which the natural promoter from the genes *guaA* or *guaB* is exchanged against an inducible one such as the rhamnose promoter.

In cancer therapy blocker of the purine biosynthesis pathway are already used (Bertino *et al.*, 2011; Issaeva *et al.*, 2010). To test if such substances would also affect purine biosynthesis of Bcc strains, we tested the drug 6-thioguanine. However, no inhibition of growth could be observed. Maybe *B. cenocepacia* H111 is not able to take this compound up, or they can export it actively by the use of multidrug efflux pumps. Other purine blockers such as mecaptopurine have been described, which would be interesting to test in the future (Bertino *et al.*, 2011).

Another interesting gene identified is *aroK*, which encodes a shikimat kinase that is essential for the biosynthesis of aromatic compounds like of phenylalanine, tyrosine and tryptophan, ubiquinone and folic acid (Han *et al.*, 2007; Parish & Stoker, 2002). This pathway should be of special interest because it is a conserved pathway in plants, algae and bacteria but is absent from mammals. This makes this pathway a perfect target for nontoxic antimicrobial agents (Han *et al.*, 2007; Kishore & Shah, 1988; Roberts *et al.*, 1998). One compound which is in

use as a herbicide is the compound glyphosate from Monsanto (N-(phosphonomethyl)glycine) also known with its commercial name "Roundup". It is an inhibitor of the 5-enolpyruvylshikimate-3-phosphate synthase and showed in previous studies inhibitory effects on growth of *P. aeruginosa, E. coli, Bacillus subtilis and Bradyrhizobium japonicum* (Fischer *et al*., 1986; Norris *et al*., 2009; Zablotowicz & Reddy, 2004). Preliminery results with this herbicide have shown that it inhibits growth of *B. cenocepacia* H111. Another compound which should be tested are 6(S)-6-fluoro-shikimic acid from AstraZeneca, which blocks the biosynthesis of *p*-aminobenzoic acid, its stereoisomer 6(R)-6-fluoro-shikimic acid and 2-fluoro shikimic acid. All these shikimate analogs contain a fluorine substitution at position C2 or C6 instead of a hydrogen and all three compounds showed some inhibitory effect when tested against *Plasmodium faliciparum* (McConkey, 1999) and *E. coli* (Bornemann *et al.*, 1995; Davies *et al.*, 1994). 6(S)-6-fluoro-shikimic acid also protected mice against *P. aeruginosa* or *Staphylococcus aureus* infections (Davies *et al.*, 1994). It would also be interesting to test if flies could be treated after pricking by feeding them with these chemicals or infected *G. mellonella* can survive when the compounds are injected into the larvae.
The third pathway which would be interesting to investigate in better detail is the pyrimidine pathway because the H111-*pyrD*::Tn*5* mutant, which is deficient for the dihydroorotat dehydrogenase, showed a high attenuation in all animal models used. However, inhibitors of this enzyme have not yet been described.

In summary, we have identified several genes in *B. cenocepacia* that are critical for pathogenicity in multiple non-mammalian infection hosts. These results not only further our understanding of the virulence mechanisms used by this opportunistic pathogen, but have also led to the identification of some potential targets for the development of novel antibacterial drugs. Some of the strongly attenuated mutants identified in this study could also be of interest for the development of live vaccines (Schwager *et al.*, 2012).

## 4.2 Future work on the role of pC3 in pathogenicity of Bcc strains

After the discovery that three attenuated *B. cenocepacia* H111 mutants have lost chromosome 3 (Uehlinger dissertation) we were able to devise a method for curing chromosome 3 from most strains of the Bcc (Agnoli *et al.*, 2012). All the ΔpC3 mutants tested showed attenuation in the *C. elegans* model. The ΔpC3 strains were also found to be attenuated in *G. mellonella*. We were also able to produce an H111 cosmid library, which was screened for virulence factors using the avirulent background of *B. cenocepacia* H111ΔpC3. To this end, we transferred the cosmid by triparental mating into the *B. cenocepacia* H111ΔpC3 mutant and screened around 2000 strains for clones with increased pathogenicity in the *C. elegans* model. To our surprise only one cosmid could be identified and preliminary data suggest that the gene encoding AidA is not present on this cosmid. Due to the fact that *aidA* is on pC3 and its lack leads to attenuation in the *C. elegans* model, it could be that some virulence genes are missed in this infection model. Therefore, the library should also be tested in the *G. mellonella* model. Because it would be extremely tedious to test 2000 strains individually, strains should be pooled before infecting the larvae. The cosmid identified in this work seems not to be attenuated when tested in *G. mellonella*, but it would be worthwhile to perform additional phenotypic and genetic tests to identify the gene(s) responsible for *C. elegans* killing.

Partial deletions of a non-essential region of chromosome three (Agnoli, unpublished) showed no effect on virulence when compared to the wild type in *C. elegans* and *D. melanogaster*. This indicates that the genes responsible for pathogenicity on pC3 must be on the remaining part of pC3. Further partial deletions of pC3 are currently constructed in our laboratory which will be tested for pathogenicity in multiple infection hosts. These experiments will identify, or at least narrow down the localization of virulence factors present on pC3.

# 5 Contribution to publications included in my thesis

## 5.1 Schwager, S., Agnoli, K., Köthe, M., Friederike, F., Givskov, M., Carlier, A. & Eberl, L. (2012). Identification of *Burkholderia cenocepacia* H111 virulence factors using non-mammalian infection hosts. *Infection and Immunity, published online ahead of print on 22 October 2012*.

- *C. elegans* pathogenicity assays
- *D. melanogaster* pathogenicity assays
- *G. mellonella* pathogenicity assays
- Construction of a cosmid library and complementation of the Tn*5* mutants
- Investigation of the purine pathway
- Composition of the script together with Kirsty Agnoli and Leo Eberl
- The construction of the Tn*5*-library, *C. elegans* screening, investigation of the protease activity, the expression of AidA, the growth on minimal medium, the AHL-, EPS- and siderophore production was done by Manuela Köthe and Friederike Feldmann.

## 5.2 Lore, N. I., Cigana, C., De Fino, I., Riva, C., Juhas, M., Schwager, S., Eberl, L. & Bragonzi, A. (2012). Cystic fibrosis-niche adaptation of *Pseudomonas aeruginosa* reduces virulence in multiple infection hosts. *PloS one* 7, e35648.

- *D. melanogaster* pathogenicity assays
- Determination of the $LD_{50}$ in *G. mellonella*

## 5.3 Agnoli, K., Schwager, S., Uehlinger, S., Vergunst, A., Viteri, D. F., Nguyen, D. T., Sokol, P. A., Carlier, A. & Eberl, L. (2012). Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid. *Molecular microbiology* 83, 362-378.

- *G. mellonella* pathogenicity assays
- *C. elegans* patogenicity assays

**5.4 Uehlinger, S., Schwager, S., Bernier, S. P., Riedel, K., Nguyen, D. T., Sokol, P. A. & Eberl, L. (2009). Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains by using multiple infection hosts. *Infection and Immunity* 77, 4102-4110.**

- *G. mellonella* pathogenicity assays

**5.5 Steindler, L., I. Bertani, L. De Sordi, S. Schwager, L. Eberl, and V. Venturi. (2009). LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants. Applied and Environmental Microbiology 75:5131-5140.**

- *G. mellonella* pathogenicity assays
- *C. elegans* pathogenicity assays

**5.6 Sousa, S. A., Ramos, C. G., Almeida, F., Meirinhos-Soares, L., Wopperer, J., Schwager, S., Eberl, L. & Leitao, J. H. (2008). *Burkholderia cenocepacia* J2315 acyl carrier protein: A potential target for antimicrobials' development? *Microbial Pathogenesis* 45, 331-336.**

- Microscopic investigation of *C. elegans* gut colonisation by the *B. cenocepacia* J2315 wild-type and mutant SJ1

**5.7 Schwager, S., Lumjiaktase, P., Stoeckli, M., Weisskopf, L. & Eberl, L. (2012). The genetic basis of cadmium resistance of *Burkholderia cenocepacia*. *Environmental Microbiology Reports* 4.**

- Majority of the experimental work, together with Martina Stöckli and Putthapoom Lumjiaktase.
- Composition of the script together with Laure Weisskopf, Putthapoom Lumjiaktase and Leo Eberl.

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# 6 References

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# 7 Appendix

# Appendix 1

## Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid

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# Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid

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## Summary

**The *Burkholderia cepacia* complex (Bcc) consists of 17 closely related species of opportunistic bacterial pathogens, which are particularly problematic for cystic fibrosis patients and immunocompromised individuals. Bcc genomes consist of multiple replicons, and each strain sequenced to date has three chromosomes. In addition to genes thought to be essential for survival, each chromosome carries at least one rRNA operon. We isolated three mutants during a transposon mutagenesis screen that were non-pathogenic in a *Caenorhabditis elegans* infection model. It was demonstrated that these mutants had lost chromosome 3 (c3), and that the observed attenuation of virulence was a consequence of this. We constructed a c3 mini-replicon and used it to cure c3 from strains of several Bcc species by plasmid incompatibility, resulting in nine c3-null strains covering seven Bcc species. Phenotypic characterization of c3-null mutants revealed that they were attenuated in virulence in multiple infection hosts (rat, zebrafish, *C. elegans*, *Galleria mellonella* and *Drosophila melanogaster*), that they exhibited greatly diminished antifungal activity, and that c3 was required for D-xylose, fatty acid and pyrimidine utilization, as well as for exopolysaccharide production and proteolytic activity in some strains. In conclusion, we show that c3 is not an essential chromosomal element, rather a large plasmid that encodes virulence, secondary metabolism and other accessory functions in Bcc bacteria.**

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## Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely related species, sharing > 97.5% 16S rDNA sequence similarity, but only moderate genome-wide similarity (30–60% DNA–DNA hybridization) (Vandamme *et al.*, 1997; Coenye *et al.*, 2001; Vandamme and Dawyndt, 2011). The Bcc currently contains 17 defined species (Vanlaere *et al.*, 2009), which are generally found in the environment, particularly in association with plants. Representatives of all species have, however, also been isolated from humans (Vandamme and Dawyndt, 2011). Bcc species are opportunistic pathogens, and are best known for causing a highly fatal pneumonia known as cepacia syndrome in cystic fibrosis patients. The most clinically relevant Bcc species are *Burkholderia cenocepacia* and *Burkholderia multivorans*, which cause 85–97% of all Bcc infections in cystic fibrosis patients. *B. cenocepacia* infection, especially, is associated with a poor clinical outcome, although prognosis appears to be highly strain-dependent (Drevinek and Mahenthiralingam, 2010).

Bcc species are notable for their ability to metabolize a wide variety of substrates including persistent environmental toxins, and to thrive in many different environments (Coenye and Vandamme, 2003; Mahenthiralingam *et al.*, 2005). Furthermore, members of the Bcc have been shown to produce antifungal agents (Kang *et al.*, 1998; Lu *et al.*, 2009; Schmidt *et al.*, 2009), and have been used as biocontrol agents to protect plants against fungal-borne diseases such as 'damping off' (Coenye and Vandamme, 2003). However, owing to their status as opportunistic pathogens, they are no longer used for this purpose (http://www.epa.gov/fedrgstr/EPA-PEST/2004/September/Day-29/p21695.htm).

The metabolic versatility of Bcc species is attributable to their varied genomes, which with sizes ranging from 7 to 9 Mb are among the largest bacterial genomes known. The genome sequences of nine Bcc species have been completed to date (*Burkholderia ambifaria* strains AMMD and MC40-6, *Burkholderia vietnamiensis* G4, *Burkholderia lata* 383, *B. multivorans* ATCC17616, *B. cenocepacia* strains J2315, MCO-3, HI2424 and AU1054). In addition

to this, our group has assembled a draft genome of *B. cenocepacia* H111, a cystic fibrosis isolate (Geisenberger *et al.*, 2000) used as a model organism in this study. The genome of each of the sequenced Bcc strains consists of three chromosomes: chromosome 1 (c1) is 3.3–3.9 Mb, chromosome 2 (c2) is 2.4–3.6 Mb, and chromosome 3 (c3) is 0.5–1.4 Mb in size. These three replicons are considered chromosomes because they appear to carry genes essential for growth (Komatsu *et al.*, 2003; Egan *et al.*, 2005; Holden *et al.*, 2009). Additionally, each of them carries at least one rRNA operon, a criterion that is often used to distinguish between a chromosome and a plasmid (Bentley and Parkhill, 2004). In addition to the three chromosomes some Bcc strains also harbour plasmids, for example *B. cenocepacia* J2315 has one, and *B. vietnamiensis* G4 has five.

This study was initiated because we observed that three H111 Tn*5* mutants attenuated in virulence in *Caenorhabditis elegans* had lost c3, indicating that this replicon is a plasmid and not a chromosome. We used plasmid incompatibility to delete c3 from nine Bcc strains, and phenotypically characterized the mutant derivatives. We found that c3 is not only important for antifungal activity but is also required for pathogenicity in various infection hosts. In summary, our results suggest that this megaplasmid confers a competitive advantage to the organism in its natural habitat.

## Results

### *Discovery of c3-loss mutants*

In the course of a large screen of *B. cenocepacia* H111 mini-Tn*5* insertion mutants for strains attenuated in virulence in a *C. elegans* infection model, we isolated three non-pathogenic mutants (R12, R33 and R44). The transposon insertion sites in these mutants were located in different positions within c2 (data not shown). All attempts to complement the three mutants failed, and a reconstructed mutant in which the H111 gene homologous to J2315 BCAM2793 was inactivated was as pathogenic as the wild type (data not shown). Southern blot analysis excluded the possibility of the presence of a second copy of the transposon elsewhere in the chromosomes of the mutants. These results clearly showed that the loss of virulence was independent of the transposon insertion, and was the consequence of a spontaneous mutation.

We next performed microarray analyses on two of the mutants, R33 and R12, using a custom *B. cenocepacia* oligonucleotide microarray consisting of 60 base oligomers (Drevinek *et al.*, 2008b). The transcriptomes of both mutants were surprisingly different to wild type (Tables S1 and S2). Particularly striking was the apparent downregulation of all c3 genes that were expressed in the wild type under the conditions used (corresponding to approximately half the genes present on J2315 c3). To investigate whether the observed differences were due to a deletion, we analysed undigested genomic DNA from the transposon mutants by pulsed-field gel electrophoresis. This revealed the absence of the third replicon (c3) in all three mutants (data not shown).

### *Construction of c3-null Bcc strains*

Because the previously isolated c3 deletion mutants of *B. cenocepacia* H111 (R12, R33 and R44) bore transposons within their genome, a defined mutant was constructed that lacked c3, but was otherwise genetically identical to wild type. A Flp/FRT-based recombination approach (as described in the *Experimental procedures*) was used to separate the bulk of c3 from those elements required for its replication (the *parAB* and *repA* genes, the *parS* sites and DnaA-binding sites), resulting in the loss of all c3 DNA except that required for replication. The resultant approximately 12.6 kb plasmid, designated pMinic3, consisted of the c3 origin of replication fused to a modified pEX18 backbone (Fig. 1). The presence of the pEX18 backbone conferred the following properties on pMinic3: a pBBR322 origin of replication for use in *Escherichia coli*; an origin for conjugal transfer; a selectable marker for trimethoprim resistance encoded by the *dhfrII* gene; and a *sacB* gene conferring sucrose sensitivity, for plasmid curing (Hynes *et al.*, 1989). This *sacB* gene was used to induce loss of pMinic3, resulting in an unmarked H111 c3-null mutant. The absence of pMinic3 was confirmed by loss of Tp resistance, and by PCR analysis using primers targeting the c3 origin of replication. To show definitively that c3 had been lost, pulsed-field gel electrophoresis was carried out on undigested genomic material from the candidate c3-null strains and their wild-type parents (Fig. 2). Plasmid pMinic3 was found to be an extremely valuable tool for the generation of unmarked c3 deletion derivatives in other strains of the Bcc through plasmid incompatibility, a method used successfully for the deletion of plasmids from bacteria (Uraji *et al.*, 2002; Ni *et al.*, 2008; Wang *et al.*, 2011). Chromosome 3 null mutants were constructed in nine Bcc strains, representing the species *B. ambifaria, Burkholderia anthina*, *B. cenocepacia, Burkholderia pyrrocinia, Burkholderia ubonensis* and *B. vietnamiensis.* Table 1 shows the size and gene content of c3 for the sequenced strains used in this study.

### *Loss of c3 does not affect growth rate in rich medium*

The c3-null Bcc strains were subjected to a range of phenotypic tests, to look for common c3-dependent properties that might be responsible for this element's presence. It was hypothesized that c3, while not necessary for

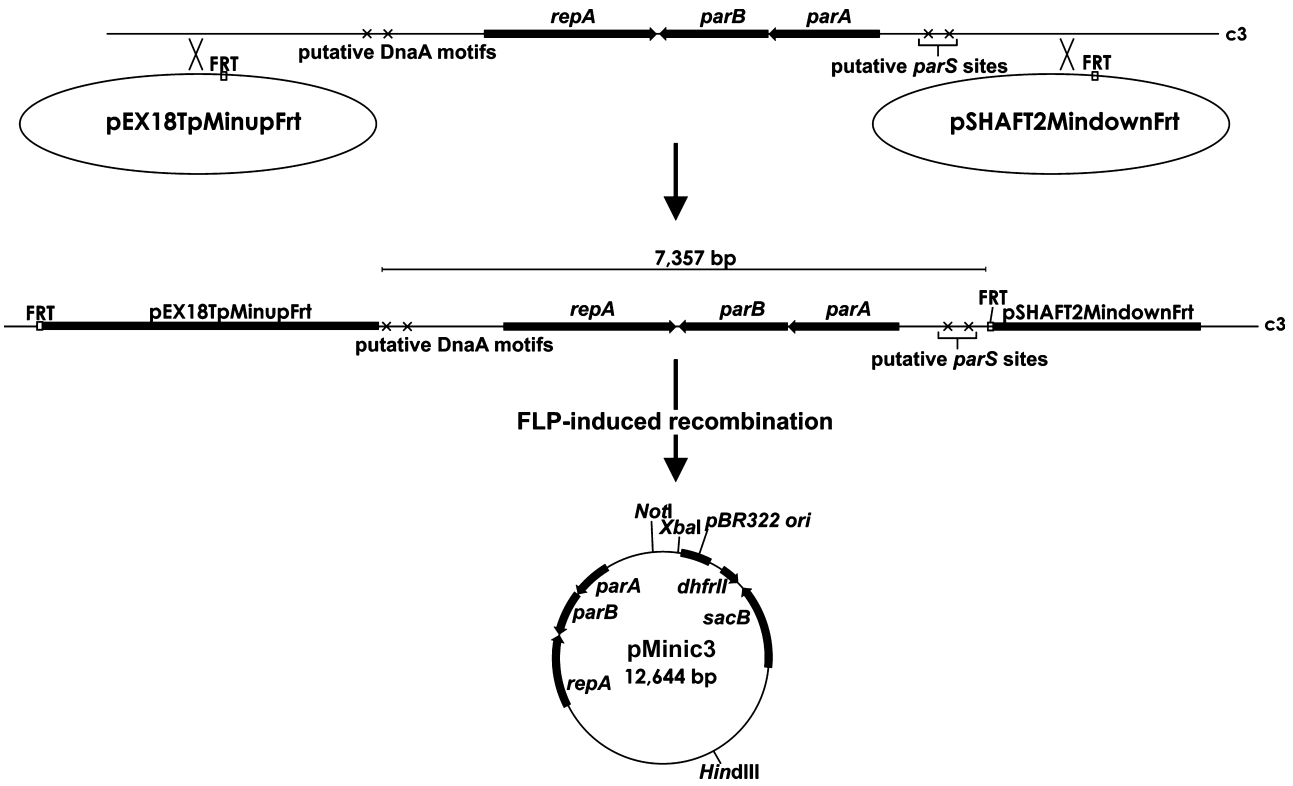


**Fig. 1.** Scheme for the construction of pMinic3. Plasmids bearing FRT sites were introduced into c3 flanking the origin of replication. Recombination at the FRT sites was stimulated by introduction of a plasmid expressing a flippase (FLP) cassette (pBBR5::FLP), giving rise to two genetic elements only one of which (pMinic3) was capable of replication. Only pertinent restriction sites are shown.

growth on rich or minimal media in the laboratory, might be necessary or advantageous for survival in environmental and clinical conditions. It was assumed that, given the non-essentiality of c3, Bcc wild-type strains would exhibit a reduced growth rate in rich medium compared with their c3 deletion derivative, owing to the additional burden of replicating, transcribing and translating c3. We tested this hypothesis using the three *B. cenocepacia* c3-null strains as models. Unexpectedly, we observed that the growth rates of *B. cenocepacia* H111, HI2424 and MCO-3 in Luria–Bertani (LB) medium were equal to those of their c3-null derivatives (Fig. S1). This demonstrated that in the rich medium used the replication, transcription and translation of c3 did not measurably limit *B. cenocepacia* growth.

### *Loss of c3 induces few changes in core metabolic functions*

To examine the metabolic functions encoded by c3, we tested the nine Bcc c3-null strains along with their wild-type parents using Biolog plate assays. The detailed results are depicted in Table S3. The absence of c3 affected relatively few of the substrate utilization phenotypes tested. In *B. cenocepacia* H111, utilization of only 13 carbon and 8 nitrogen sources of the 288 tested differed between wild-type and the c3-null derivative. Ten of these phenotypes were conserved in the three *B. cenocepacia* strains tested: D-xylose, thymidine, caproic acid, L-isoleucine, citraconic acid and gelatin as C-sources; uracil, thymine, thymidine and histamine as N-sources. Some phenotypes were found to be dependent on c3 in most of the Bcc strains. For example, the utilization of caproic acid as a carbon source required c3 in all strains tested except for *B. vietnamiensis* LMG10929. Inspection of the available Bcc genome sequences revealed candidate genes on c3 for some of the most commonly observed c3-dependent phenotypes. As shown in Table 2, three of the enzymes necessary for β-oxidation (thiolase, β-hydroxyacyl-CoA dehydrogenase and acyl-CoA dehydrogenase) appear to be encoded on c3 in the sequenced Bcc members. These enzymes are required for metabolism of fatty acids such as caproic, capric and butyric acid. D-xylose utilization as a carbon source was dependent on c3 in all strains except for *B. vietnamiensis* LMG10929 and *B. lata* 383. A gene cluster probably responsible for D-xylose utilization was identified on c3, consisting of genes putatively encoding a xylose isomerase, a D-xylose-binding periplasmic protein precursor, a D-xylose ABC transporter ATP-binding protein and a xylose permease (Table 2). The use of uracil as a

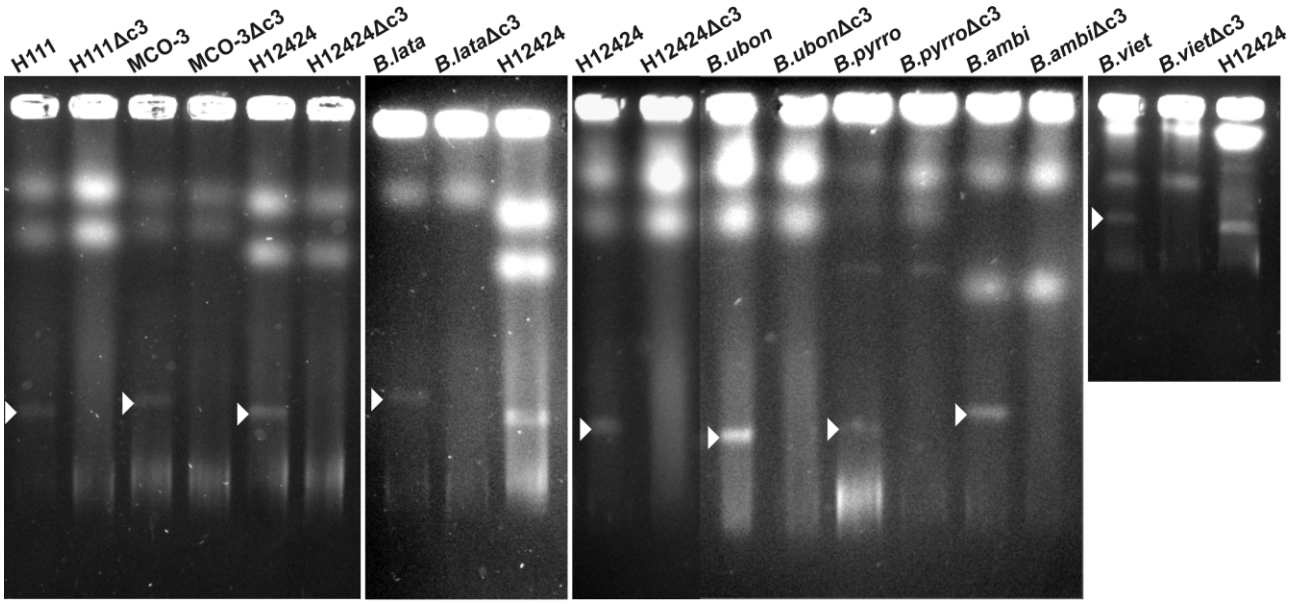


**Fig. 2.** Visualization of undigested genomic DNA from wild-type Bcc strains and their c3-null mutants. Genomic DNA was separated by pulsed-field gel electrophoresis. Chromosome 3 has been indicated for each wild-type strain using arrowheads. Abbreviations are as follows; H111: *B. cenocepacia* H111; H111Δc3: *B. cenocepacia* H111Δc3; MCO-3: *B. cenocepacia* MCO-3; MCO-3Δc3: *B. cenocepacia* MCO-3Δc3; HI2424: *B. cenocepacia* HI2424; HI2424Δc3: *B. cenocepacia* HI2424Δc3; *B. lata: B. lata* 383; *B. lata*Δc3: *B. lata* 383Δc3; *B. ubon: B. ubonensis* LMG20358; *B. ubon*Δc3: LMG20358Δc3; *B. pyrro: B. pyrrocinia* LMG14191, *B. pyrro*Δc3: *B. pyrrocinia* LMG14191Δc3*; B. ambi: B. ambifaria* AMMD; *B. ambi*Δc3*: B. ambifaria* AMMDΔc3; *B. viet: B. vietnamiensis* LMG10929; *B. viet*Δc3*: B. vietnamiensis* LMG10929Δc3. *B. cenocepacia* HI2424 was included on each gel as a standard, as the size of each replicon is known for this strain. The contrast in the third panel from the left has been manipulated in two sections, so as to maximize visibility of the replicons. It was necessary to use a different separation procedure for *B. vietnamiensis* LMG10929 to reduce smearing (see *Experimental procedures*). *B. anthina* LMG20983 and its c3-null counterpart have not been included, as the replicons of these strains could not be successfully visualized.

nitrogen source was c3-dependent in all strains except *B. ambifaria* AMMD and *B. vietnamiensis* LMG10929. Enzymes potentially involved in the pyrimidine metabolic and the pantothenate and CoA biosynthetic pathways were found to be encoded on c3. These genes were probably responsible for the c3-dependence of thymine, thymidine and uracil utilization (Table 2). We exploited the most common c3-dependent phenotypes in the design of a medium selective for the presence of c3. It was found that wild-type Bcc grew very poorly, if at all, when D-xylose or caproic acid were used as the carbon source in minimal media. However, a glucose-containing minimal medium supplemented with uracil as the sole nitrogen source was found to be selective against c3-null mutants.

**Table 1.** Genome statistics for sequenced Bcc strains.

| Strain | Total genome (Mb) | c3 (Mb) | Gene content of c3 |
|---|---|---|---|
| *B. lata* 383 | 8.68 | 1.40 | 1209 |
| *B. vietnamiensis* G4[a] | 8.39 | 1.24 | 1114 |
| *B. ambifaria* AMMD | 7.53 | 1.28 | 967 |
| *B. cenocepacia* H111 | 7.72 | 1.04 | 965 |
| *B. cenocepacia* MCO-3 | 7.97 | 1.22 | 1053 |
| *B. cenocepacia* HI2424 | 7.70 | 1.06 | 918 |

**a.** The *B. vietnamiensis* strain used in this study has not been sequenced.

### *EPS production is influenced by c3 in some Bcc species, but biofilm formation is unaffected*

Biofilm formation is not only crucial for chronic infections, but also for persistence in the environment. One of the key factors in biofilm formation is the biosynthesis of extracellular polymeric substances (EPS) (Riedel and Eberl, 2007). The production of EPS by the c3-null mutants and their wild-type parents was examined by streaking the bacteria on mannitol plates, which are known to stimulate EPS production in Bcc strains (Zlosnik *et al.*, 2008). A large difference in EPS production was observed in the cases of *B. pyrrocinia* LMG14191 and *B. ambifaria* AMMD, with the wild-type strains producing much more EPS than their c3-null derivatives. There was no obvious difference in EPS production between the other strains and their c3 deletion derivatives (see Fig. 3). Those *Burkholderia* EPS clusters that have been characterized to date (the *bce* and *wcb* clusters, and the EPS transporter cluster putatively encoded by Bamb_3621–3629) are encoded on *B. ambifaria* AMMD c1 and c2 (Bartholdson *et al.*, 2008). However, the c3-encoded Bamb_6177 gene contains a domain

**Table 2.** Genes putatively involved in the most common c3-dependent phenotypes as determined by phenotypic microarray.

| c3-dependent phenotype | Genes likely to be involved in each sequenced strain | Comments |
|---|---|---|
| Pyrimidine utilization | J2315 (GeneID: 6804050–6804054, 6803969, 6803970,6803974)<br>MCO-3 (GeneID: 6125639-6125632)<br>HI2424 (GeneID: 4454310–4454317)<br>383 (GeneID: 3733976–3733983)<br>AMMD (GeneID: 4314867–4314871)<br>MC40-6 (GeneID: 6181971, 6181972, 6181905–6181907)<br>ATCC17616 (GeneID: 5770643–5770645, 5770511, 5770512)<br>G4 (GeneID: 4948413–4948417) | This cluster putatively encodes the enzymes for thymine, thymidine and uracil utilization (EC2.4.2.4, EC1.3.1.2, EC3.5.2.2, EC3.5.1.6) |
| Fatty acid utilization | J2315 (GeneID: 6804056–6804058)<br>HI2424 (GeneID: 4454306–4454308)<br>MCO-3 (GeneID: 6125628–6125630)<br>383 (GeneID: 3733972–3733974)<br>MC40-6 (GeneID: 6181909–6181911) AMMD (GeneID: 4314873–4314875)<br>ATCC17616 (GeneID: 5770615, 5771080, 5771081)<br>G4 (GeneID: 4948409–49448411) | These putatively encode three of the enzymes necessary for β-oxidation (thiolase, β-hydroxyacyl-CoA dehydrogenase, acyl-CoA dehydrogenase). |
| D-xylose utilization | J2315 (GeneID: 6803995–6804248)<br>MCO-3 (GeneID: 6125254–6126003), H12424 (GeneID: 4453873–4453879)<br>AMMD (GeneID: 4315234–4315239)<br>M40-6 (GeneID: 6181847–6181853)<br>G4 (GeneID: 4949351–4949357) | These putatively encode xylose isomerase, a D-xylose-binding periplasmic protein precursor, a D-xylose ABC transporter ATP-binding protein, and a xylose permease. |

typical of EPS transporters. The loss of this gene may contribute to the reduction in EPS production observed in the c3-null mutant.

To investigate whether c3 loss resulted in a reduction in biofilm production, a crystal violet microtitre-plate assay was carried out to quantify biofilm production by the c3-null mutants and their wild-type parents. Biofilm production was not affected by loss of c3 in any of the strains tested (data not shown).

### *c3 encodes antifungal agents*

In all of the Bcc strains tested in which the wild type showed activity against *Rhizoctonia solani* and/or *Fusarium solani*, deletion of c3 reduced or abrogated this activity (Fig. 4). This demonstrates that c3 encodes factors involved in the production of antifungal compounds. In agreement with our data, several gene clusters known to encode antifungals have been found on c3. For example, *B. ambifaria* AMMD

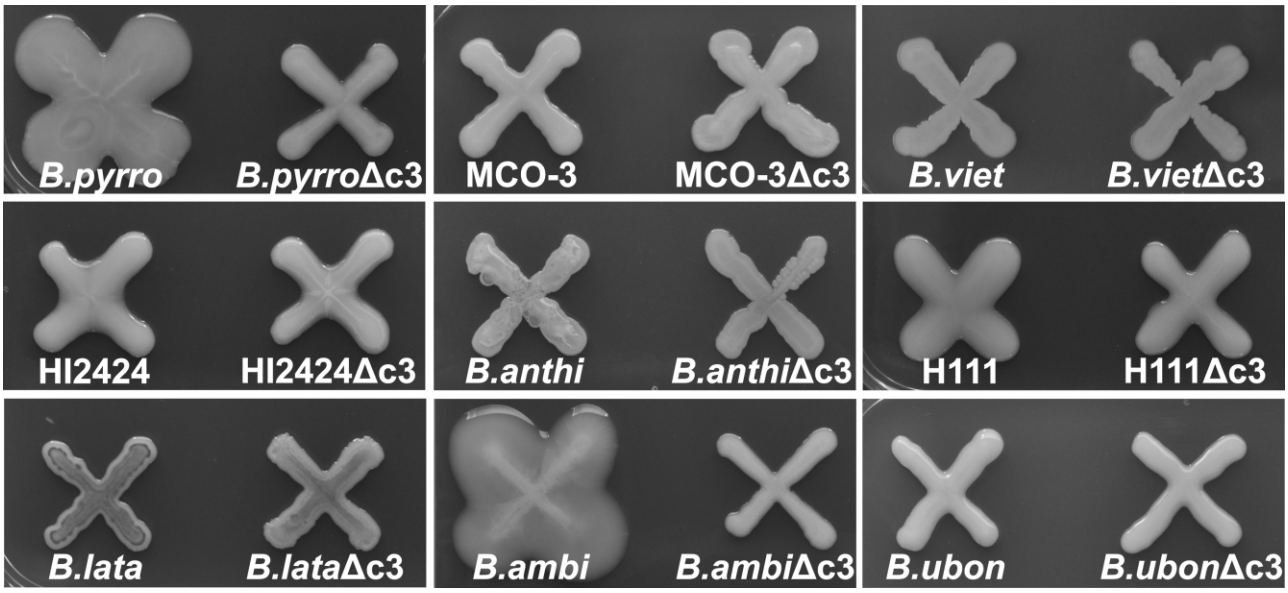


**Fig. 3.** EPS production by wild-type Bcc strains and their c3 deletion derivatives. Overnight cultures were streaked on mannitol agar and grown at 30°C for 3 days, and then at room temperature for a further 2 days. Each photograph shows wild-type (left) and its c3-null derivative (right). Strain abbreviations are as in Fig. 2, with the addition of *B. anthi: B. anthina* LMG20983 and *B. anthi* Δc3: *B. anthina* LMG20983Δc3.

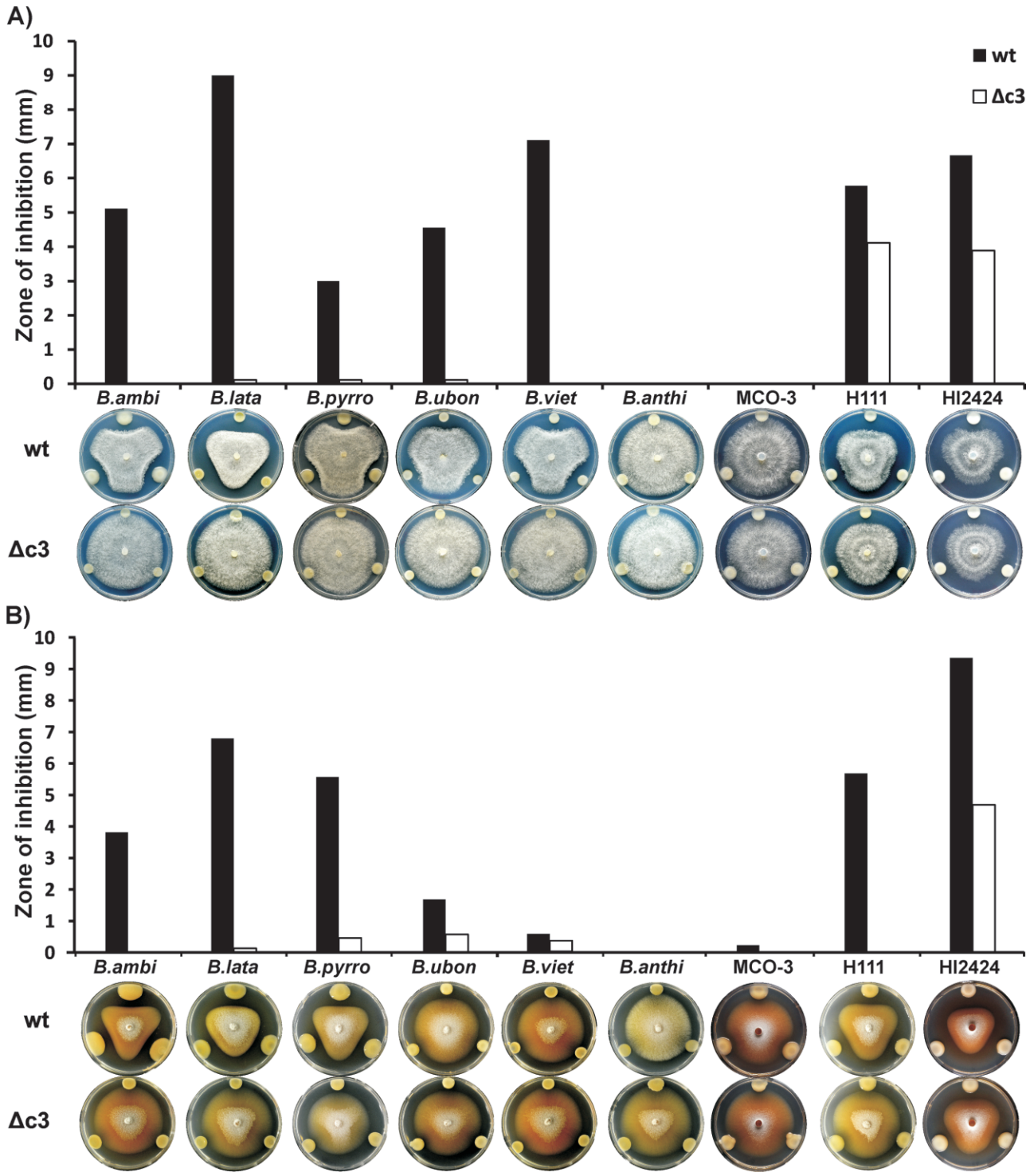


**Fig. 4.** Antifungal activity of wild-type Bcc strains (black bars) and their c3-null derivatives (white bars). Bars represent the mean zone of inhibition surrounding the bacteria. Strain abbreviations are as in Fig. 3. The assay was performed in triplicate.
A. activity against *Rhizoctonia solani,* plates incubated at room temperature for 3 days.
B. activity against *Fusarium solani,* plates incubated at room temperature for 9 days. Representative plates are shown for each result.

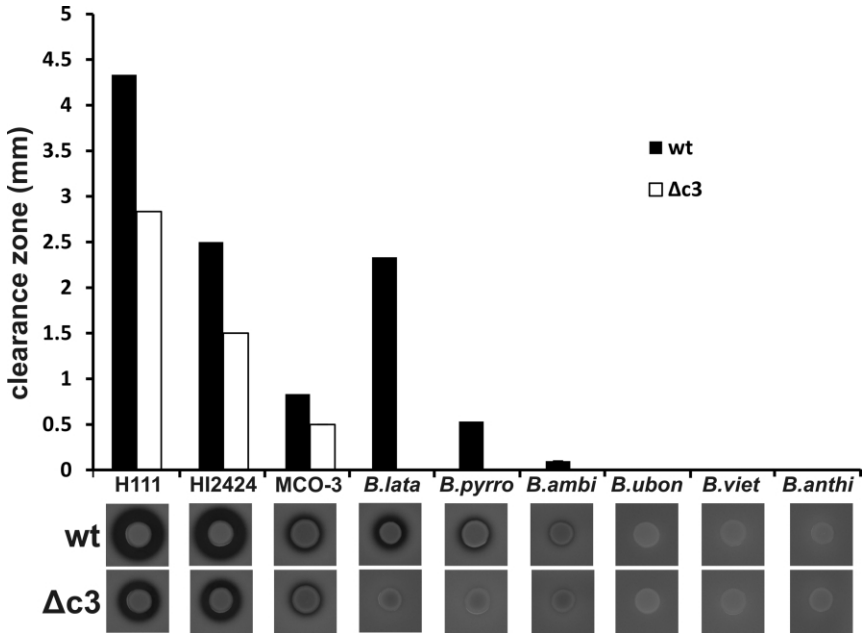


**Fig. 5.** Proteolytic activity of wild-type Bcc strains (black bars) and their c3-null derivatives (white bars). Protease activity was assayed using 2% skimmed milk agar plates. The width of the clearance zones surrounding the bacteria was measured. Each bar shows the mean of three tests. Representative photographs are shown underneath the corresponding strain names. Strain abbreviations are as in Fig. 3.

c3 carries the following clusters encoding antifungal agents: the occidiofungin cluster, which is homologous to that known to direct the biosynthesis of the antifungal glycopeptide occidiofungin in *Burkholderia contaminans* (Lu *et al*., 2009) (GeneIDs: 4315369–4315386); the *haq* genes (GeneIDs: 4314670–4314676), responsible for the production of 4-hydroxy-2-alkylquinolines, some of which have intrinsic antifungal activity (Moon *et al*., 1996; Vial *et al*., 2008); and a biosynthetic island for the production of an enacyloxin (GeneIDs: 4314815, 4314824, 4314826, 431484829–31, 4314833, 4314844) (Mahenthiralingam *et al*., 2011). The *prnABCD* operon, which directs the biosynthesis of pyrrolnitrin, is located on c3 in *B. lata* 383. As pyrrolnitrin is particularly active against *R. solani,* this might explain the loss of activity against this fungus in the c3 cured strain. Finally, the *afc* gene cluster, which encodes a lipopeptide antibiotic with strong antifungal activity (Kang *et al*., 1998), is present on c3 in the sequenced *B. cenocepacia* H111, J2315 (GeneIDs: 6804125, 6804126, 6804595, 6804594), HI2424 (GeneIDs: 4454746–4454749), MCO-3 (GeneIDs: 6125414–6125417), Au1054 (GeneIDs: 4096689–4096692), *B. lata* 383 (GeneIDs: 3734010–3734013), and *B. ambifaria* AMMD (GeneIDs: 4315053–4315056) genomes. Hence, the loss of this cluster is likely to be responsible for the observed reduction in antifungal activity in several Bcc c3 deletion derivatives (Bernier *et al*., 2008; O'Grady *et al*., 2011).

### *Extracellular proteolytic activity is influenced by the presence of c3*

Proteases are among the virulence factors of *B. cenocepacia*: both the ZmpA and ZmpB metalloproteases (J2315 GeneIDs: 6804294, 6929882) have been shown to contribute to the virulence of the organism in a rat agar bead model of infection (Corbett *et al*., 2003; Kooi *et al*., 2006). In all strains that exhibited proteolytic activity, i.e. *cenocepacia* strains H111, HI2424 and MCO-3, *B. lata* 383 and *B. ambifaria* AMMD, loss of c3 resulted in reduced activity, as tested using skimmed milk agar plates. This was especially noticeable for *B. cenocepacia* strains H111 and H12424, and *B. lata* 383 (Fig. 5). Homologues of the *zmpA* gene were found on c3 in *B. cenocepacia* strains H111, MCO-3 (GeneID: 6124952) and HI2424 (GeneID: 445411) and *B. lata* 383 (GeneID: 3734809), but on c2 in *B. ambifaria* AMMD (GeneID: 4312732). The *zmpB* gene was also found in all strains, but is located on c2. The *zmpA* protease was probably only responsible for part of the observed proteolytic activity in these strains, as the *zmpB* gene has been found to have greater activity against the casein present in skimmed-milk agar plates (Kooi *et al*., 2006). Because neither *zmpA* nor *zmpB* is located on *B. ambifaria* AMMD c3, the observed reduction in proteolytic activity in the c3-null mutant must be due to either loss of a c3-encoded regulatory element, or of an as-yet uncharacterized c3-encoded protease, such as Bamb_5605 (GeneID: 4314600).

### *Loss of c3 results in attenuated pathogenicity in multiple infection hosts*

To assess the role of c3 in the pathogenicity of Bcc strains, the entire panel of c3-null mutants was tested against the wild-type parents in the *C. elegans* and *Galleria mellonella* models of infection. Previous work has shown that both the nematode, *C. elegans,* and the larvae of the greater wax

**Table 3.** Pathogenicity of wild-type Bcc strains and their c3 deletion derivatives to *C. elegans*.

| Species | Strain | Appearance (day 2) | % live worms (day 2)[a] | Worms (day 5)[b] | Pathogenicity score[c] |
|---|---|---|---|---|---|
| *B. ambifaria* | LMG19182 | Normal | 95 | 100–500 | 0 |
| *B. ambifaria* | ambiΔc3 | Normal | 92 | > 500 | 0 |
| *B. anthina* | LMG20983 | Sick | 74 | 20–50 | 2 |
| *B. anthina* | anthiΔc3 | Normal | 94 | > 500 | 0 |
| *B. cenocepacia* | H111 | Sick | 56 | 1 | 3 |
| *B. cenocepacia* | H111Δc3 | Normal | 82 | 100–500 | 0 |
| *B. cenocepacia* | HI2424 | Sick | 65 | 6 | 3 |
| *B. cenocepacia* | HI2424Δc3 | Normal | 89 | 100–500 | 0 |
| *B. cenocepacia* | MCO-3 | Normal | 80 | 100–500 | 0 |
| *B. cenocepacia* | MCO-3Δc3 | Normal | 96 | > 500 | 0 |
| *B. lata* | 383 | Sick | 69 | 6 | 3 |
| *B. lata* | lataΔc3 | Normal | 94 | > 500 | 0 |
| *B. pyrrocinia* | LMG14191 | Normal | 80 | 50–100 | 0 |
| *B. pyrrocinia* | pyrroΔc3 | Normal | 93 | 100–500 | 0 |
| *B. ubonensis* | LMG20358 | Sick | 67 | 20–50 | 3 |
| *B. ubonensis* | ubonΔc3 | Normal | 77 | 100–500 | 0 |
| *B. vietnamiensis* | LMG10929 | Normal | 87 | > 500 | 0 |
| *B. vietnamiensis* | vietΔc3 | Normal | 94 | > 500 | 0 |

**a.** Percentage of the original number of worms alive on day 2. Number given is the mean of three separate experiments.
**b.** Number of worms alive on the plates at day 5. Number given is the mean of three separate experiments.
**c.** Pathogenicity score was calculated as described in *Experimental procedures* section.

moth, *G. mellonella,* are very valuable models for studying the pathogenesis of *Burkholderia* species (Huber *et al*., 2004; Cardona *et al*., 2005; Seed and Dennis, 2008; Uehlinger *et al*., 2009). The pathogenicity scores for *C. elegans* are shown in Table 3. For each wild-type strain exhibiting pathogenicity, the c3-null derivative was found to be non-pathogenic. This attenuation can be at least partly attributed to the loss of the c3-encoded protein AidA, which was previously shown to be essential for slow killing of nematodes by *B. cenocepacia* H111 (Huber *et al*., 2004).

Figure 6 shows the percentage survival of *G. mellonella* larvae infected with wild-type and c3-deleted strains. In most cases the c3-deleted strains were significantly less pathogenic (using the Breslow generalized Wilcoxon test) than their wild-type parents, except for *B. cenocepacia* MCO-3, *B. vietnamiensis* LMG10929, *B. lata* 383 and *B. anthina* LMG20983 (data not shown). Wild-type *B. anthina* LMG20983 showed little pathogenicity in this model, and therefore it was unsurprising that there was no significant difference in this case. Unlike in the *C. elegans* model, the c3-deleted strains retained a low level of pathogenicity in *G. mellonella*, except in the case of *B. cenocepacia* H111Δc3.

We also tested *B. cenocepacia* strain H111Δc3 in the alfalfa and rat models of infection, and HI2424Δc3 in the alfalfa, rat and zebrafish models. The *B. cenocepacia* strains tested in these models were chosen on the basis of their pathogenicity to the different infection hosts. *B. cenocepacia* strain MCO-3, for example, was found to have little pathogenicity in these models, and was therefore excluded from further tests.

The zebrafish embryo has recently been established as a novel model for the investigation of Bcc virulence (Vergunst *et al*., 2010). Although an adaptive immune system does not arise until 3 weeks after fertilization, an innate immune system resembling that of humans is already developing in the young embryo (Meijer and Spaink, 2011). A major advantage of the model is the transparency of the embryo, which allows real-time analysis of infection with fluorescently labelled bacteria. Using this animal model, *B. cenocepacia* K56-2 has been shown to create an intracellular replication niche in macrophages, followed by the development of a systemic fatal infection (Vergunst *et al*., 2010). *B. cenocepacia* strain H111 was found to be non-pathogenic in zebrafish, and therefore strain HI2424, which killed on average 43% of the embryos by 67 h post infection (p.i.), was used instead. Interestingly, infection with the c3-null mutant resulted in highly attenuated virulence compared with its HI2424 parent, confirmed by the absence of bacterial growth, a 98% embryo survival rate and real-time analysis (Figs 7 and 8). Although we did not observe any increase in the number of mutant bacteria in the embryos, the embryos had not eradicated the bacteria by 70 h p.i. (not shown), suggesting a persistent infection could be established.

Several Bcc species are pathogenic to plants. *B. cenocepacia* strains HI2424 and H111 were found to be virulent in the alfalfa model (Bernier *et al*., 2003), with almost all

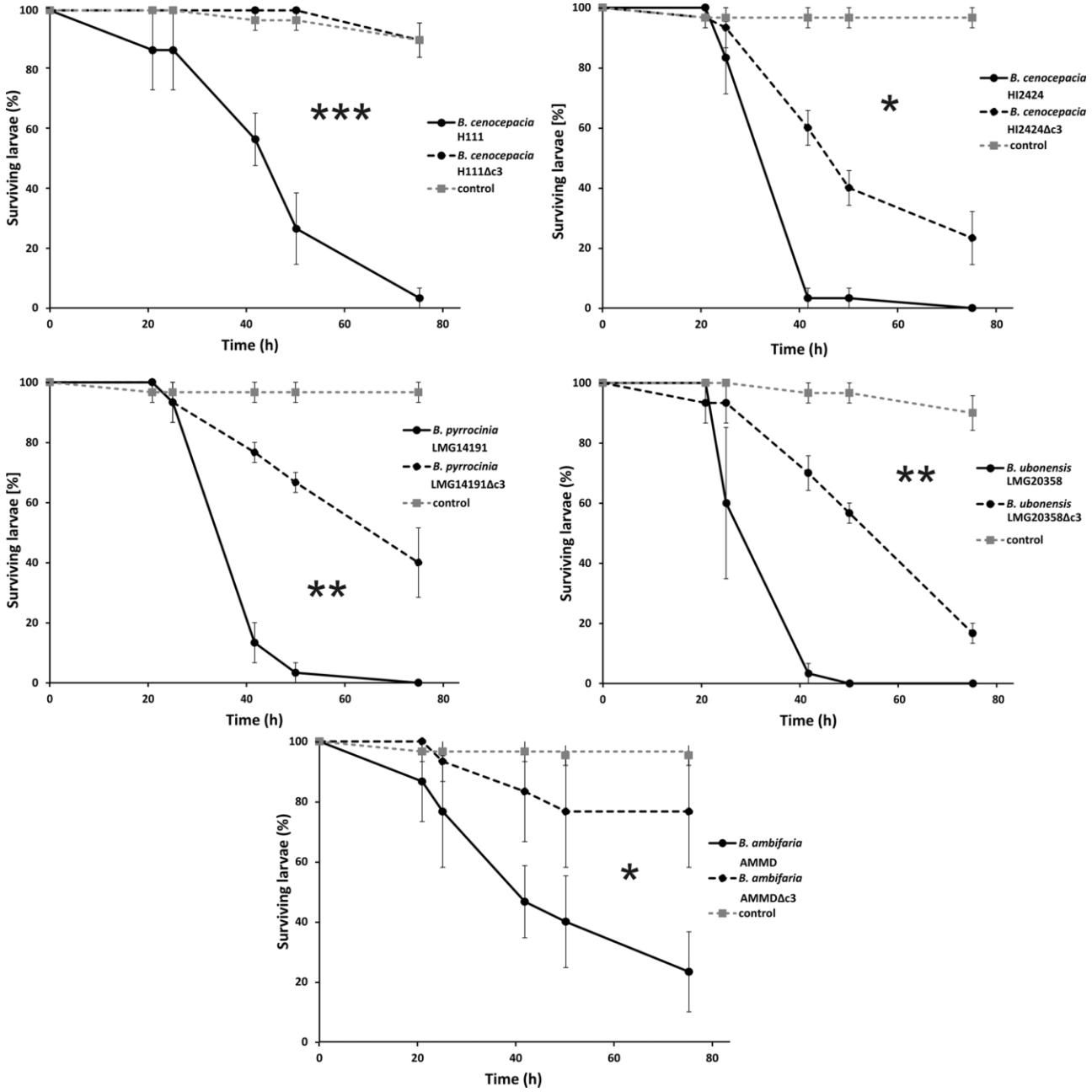


**Fig. 6.** Survival curves for *G. mellonella* larvae infected with wild-type Bcc strains and their c3 deletion derivatives. Larvae were injected with approximately $7.5 \times 10^4$ bacteria and incubated at 30°C in the dark. Live and dead larvae were counted at 20, 24, 40, 48 and 72 h p.i. Curves represent the mean of three separate experiments. Error bars represent the standard deviation of the data. All nine c3-null mutants were tested in parallel with their wild-type parents. Significance was determined using the Breslow generalized Wilcoxon method, and is indicated as follows: *$0.01 \leq P \leq 0.05$; **$0.001 \leq P \leq 0.01$, ***$P < 0.001$.

plants showing a severe phenotype 7 days after infection with the wild-type strains. In contrast, only approximately 15% of HI2424Δc3 infected seedlings died, and H111Δc3 was avirulent in this model (see Fig. S2).

Rats were infected with *B. cenocepacia* H111 or the c3-null transposon mutant R33, and lungs were removed 7 days p.i. for quantification of both the number of bacteria persisting in the lungs and the degree of inflammation, as determined by quantificating histopathological changes. There were no differences between H111 and R33 in their ability to establish a chronic infection and to persist for at least 7 days p.i. (Fig. 9A); however, there was a 41% decrease in the mean percentage of the lungs sections with inflammatory exudates (Fig. 9B). Therefore, although

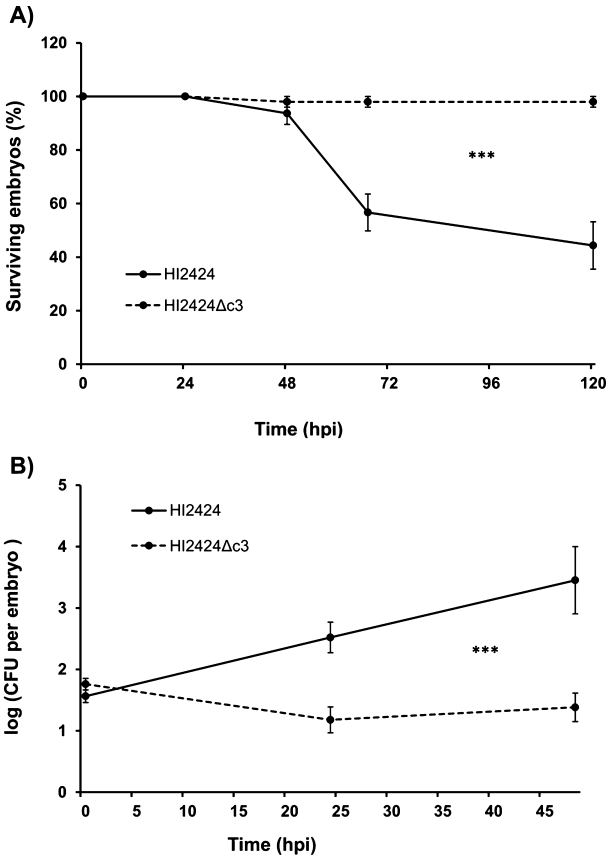


**Fig. 7.** Pathogenicity of *B. cenocepacia* HI2424 and its c3-null derivative in the zebrafish embryo. Embryos were microinjected with 20–200 cfu into the circulatory system, and incubated at 28°C in E3 medium. Embryos were randomized, and subgroups were used to determine mortality rate (A) or bacterial multiplication (B). Curves represent the mean of three separate experiments. Error bars represent the standard error of the mean.
A. Survival curves for zebrafish embryos infected with *B. cenocepacia* HI2424 and its c3-null derivative. Dead embryos were counted at 24, 48, 67 and 120 h p.i. Significance was determined using the Breslow generalized Wilcoxon method (see *Experimental procedures* for details), and was found to be $< 0.001$. Number of analysed embryos per strain was between 14 and 23 per experiment.
B. Bacterial multiplication within embryos. At each time point, five embryos were individually analysed per strain per experiment. Significance was determined using ANOVA.

similar numbers of bacteria were present in the lungs, the pathology that occurred was significantly less ($P < 0.02$) in animals infected with R33 compared with H111. Lung infection experiments were also performed with HI2424 and its c3-null derivative. As with H111, both strains were able to establish a chronic infection, and there was no difference in the number of bacteria recovered from infected animals on day 7 p.i., with approximately $10^5$ cfu recovered from 2–3 lungs in each group. In lungs examined for histopathological changes, there was a 64% decrease in the mean percentage of lungs infiltrated with inflammatory exudate (Fig. 9C), although in this experiment the difference between HI2424 and the c3 deletion mutant was not quite significant ($P = 0.08$). The trend observed in infections with these two strains and their corresponding mutants supports a role for c3-encoded genes in virulence in lung infections.

## Discussion

To date, c3 has been considered a secondary chromosome for four reasons: (i) it has been described to carry essential genes (Komatsu *et al.*, 2003; Egan *et al.*, 2005; Holden *et al.*, 2009) although, to our knowledge, solid experimental proof for this statement is not available, (ii) it carries ribosomal RNA genes (Rodley *et al.*, 1995; Lessie *et al.*, 1996), (iii) it shows a GC skew typical for a chromosome (Dubarry *et al.*, 2006; Holden *et al.*, 2009) and (iv) all Bcc strains sequenced to date (> 20 strains) carry c3. Here we have shown that c3 is not essential for growth under standard laboratory conditions; c3 should therefore be reclassified as a megaplasmid, and we suggest naming it pC3. While c1 has a bacterial chromosome-type origin of replication, as evidenced by the presence of *gidA, dnaA, rpmH, rnpA* and *gyrB* genes, c2 and pC3 have origins typical of low copy number plasmids, each bearing a *repA* gene encoding a replication initiator/control protein. It has therefore been proposed that both c2 and pC3 have originated from plasmids (Dubarry *et al.*, 2006). The presence of genes encoding ribosomal RNAs is commonly interpreted to be indicative of a chromosome (Bentley and Parkhill, 2004). The only exception reported thus far is *Ralstonia solanacearum*, which harbours a 2.1 Mb second replicon that carries, in addition to a complete copy of an rRNA locus with two tRNA genes, genes coding for the α-subunit of DNA polymerase III and for protein elongation factor G. This second replicon was designated a megaplasmid because it is probably unnecessary for survival. Whether or not this replicon is truly a plasmid is still an open question, as a megaplasmid-deleted *Ralstonia* strain has not yet been constructed (Salanoubat *et al.*, 2002).

Surprisingly, in the microarray analysis of the two *B. cenocepacia* H111 pC3-null transposon mutants, R12 and R33, only 55 of the genes present on c1 or c2 were found to be more than threefold up- or down-regulated compared with H111 (Table S2). This suggests that relatively little regulatory cross-talk took place between pC3 and the chromosomes. Some of the c1 and c2 genes that showed a large (> fivefold) difference in expression are regulated by the CepI/R quorum sensing system, perhaps via the pC3-encoded ShvR regulator (Table S2) (O'Grady *et al.*, 2009). This regulator has been shown to influence expression of various quorum sensing-regulated genes and to be important for antifungal and proteolytic activity in *B. cenocepacia* K56-2 (Bernier *et al.*, 2008; O'Grady *et al.*, 2011).

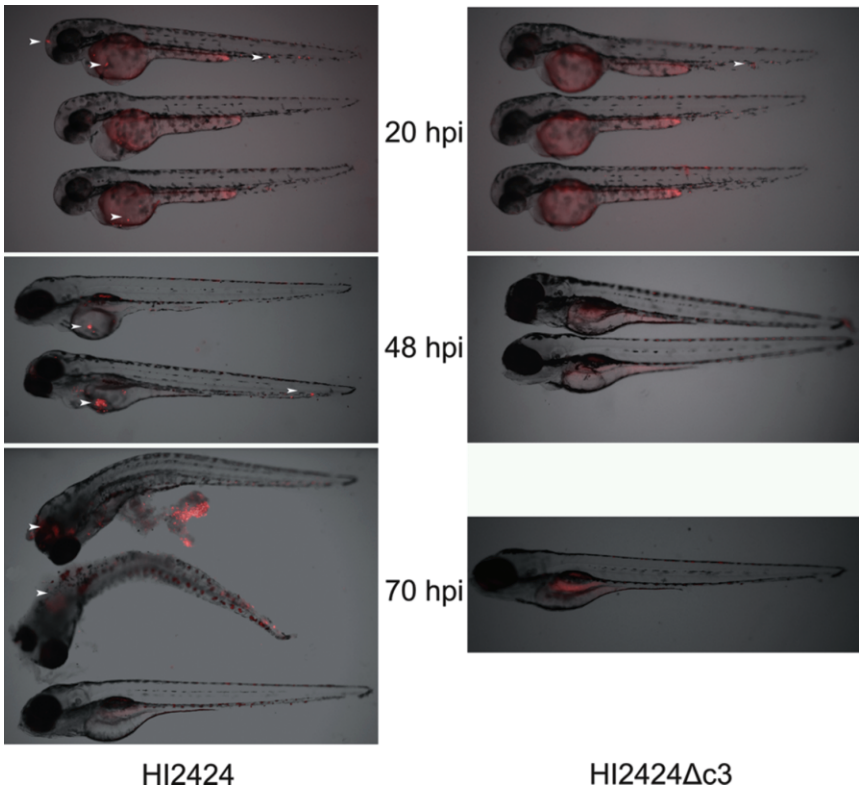


**Fig. 8.** Real-time visualization of *B. cenocepacia* HI2424 and HI2424Δc3 in infected zebrafish embryos. Zebrafish embryos were inoculated with between 20 and 200 cfu of ds-red-expressing HI2424 or HI2424Δc3. The *B. cenocepacia* strain used for infection is indicated below the photographs, and the time p.i. is given between the images. Typical infected macrophages (20 hpi), local infection sites (48 hpi), and embryos showing systemic infection with massive bacterial multiplication and tissue inflammation for HI2424 but not for the c3 deletion mutant (70 hpi) are indicated with white arrow heads. Representative images from one of four independent experiments are shown.

Given the non-essentiality of pC3, it seems that strong selective pressures for its maintenance must exist in the natural environment, which are absent from the laboratory. In support of this, Yoder-Himes and colleagues found that most genes specifically induced in sterile soil or soil extract were located on c2 and pC3 (Yoder-Himes *et al.*, 2009; Nishiyama *et al.*, 2010). Highly conserved pC3-dependent metabolic phenotypes include D-xylose utilization, fatty acid metabolism and pyrimidine utilization. The substrates for these catabolic pathways may be available in the Bcc's typical niche in the plant rhizosphere, because D-xylose, fatty acids and pyrimidines are typical components of decaying matter.

The production of EPS was found to be pC3-dependent in *B. pyrrocinia* LMG14191 and *B. ambifaria* AMMD, with much more EPS produced by the wild-type strains than their pC3-null derivatives. Although EPS is one of the main constituents of a biofilm, we did not observe any defects in biofilm production by *B. pyrrocinia* and *B. ambifaria* pC3-null mutants relative to their wild-type parents. This finding may indicate that the polysaccharides produced on mannitol plates are different from the one(s) that are required for biofilm formation on a plastic surface.

Plasmid pC3 is important for virulence. In the *C. elegans* and *G. mellonella* infection models, seven of the nine Bcc strains tested showed an attenuation in virulence in one or both models, indicating that pC3 plays an important role in pathogenesis of Bcc strains in non-mammalian infection hosts. The results obtained using the zebrafish and rat models indicated that pC3 is important for pathogenicity of *B. cenocepacia* in higher organisms.

Although the wild-type strains showed a wide spectrum of pathogenicity levels in the various infection hosts used, deletion of pC3 resulted in attenuated virulence in most cases. Hence, pC3 is likely to encode functions that enable Bcc strains not only to combat their natural predators in the environment, but also to infect humans. In agreement with these results, comparative genomics has revealed that c1 carries mostly housekeeping genes, while c2 and particularly pC3 encode accessory functions, including virulence determinants (Holden *et al.*, 2009). These data demonstrate that, while pC3 is not necessary for growth under laboratory conditions, it is required for pathogenicity within various host organisms.

In summary, our data provide clear evidence that pC3 shows all the characteristics of a virulence plasmid. To shed further light on the evolutionary history of plasmid pC3, we identified those genes conserved in all publicly available pC3 sequences, and in H111 pC3. Intriguingly, the Bcc pC3 plasmids shared only 65 genes, clustered around the origin of replication mostly on the leading strand (Fig. S3). To indicate whether pC3 was present in the last common ancestor of the Bcc species, as opposed to its being acquired by horizontal transfer, we constructed a phylogenetic tree using the 65 conserved pC3 genes. The topology of this tree was identical to that of

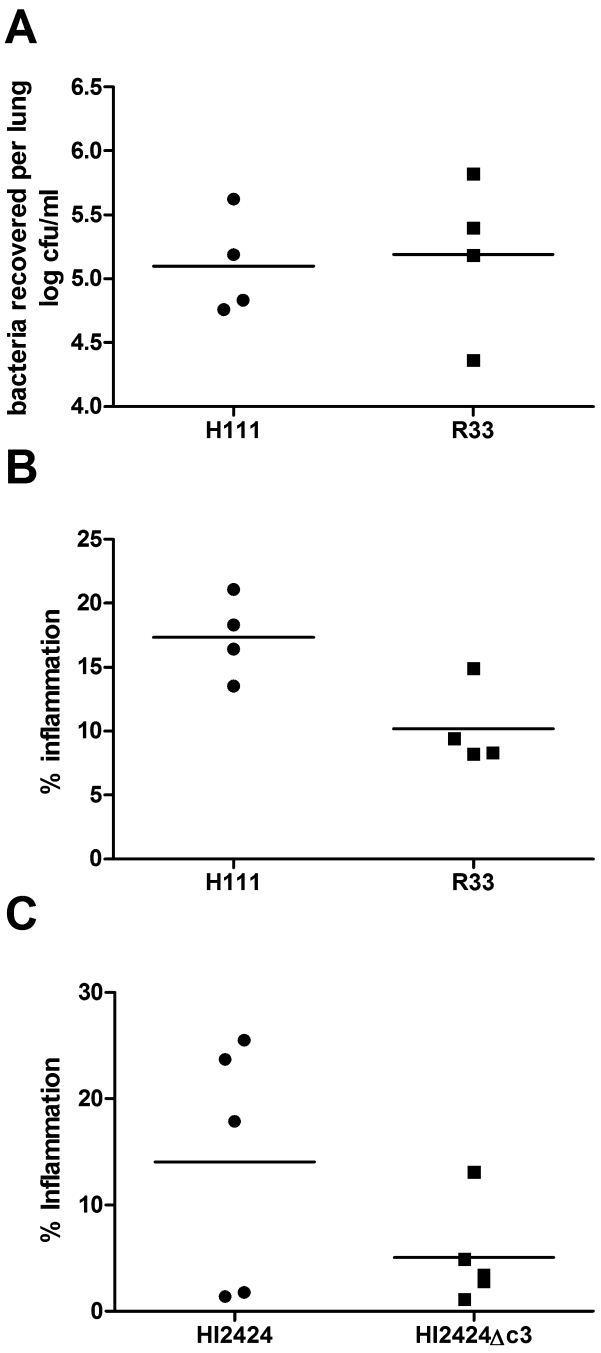


**Fig. 9.** Pathogenicity of *B. cenocepacia* strains in the rat lung chronic infection model. Rats were infected with wild-type *B. cenocepacia* or the c3-null transposon mutant, and lungs were removed 7 days p.i. for quantification of both the number of bacteria persisting in the lung and the degree of inflammation, as determined by quantifying histopathological changes in the lung. Each experiment shown was performed once.
A. cfu recovered from lungs infected with H111 and the c3-null transposon mutant R33.
B. Histopathology (% inflammation) for H111 and the c3-null transposon mutant R33.
C. Histopathology (% inflammation) for HI2424 and the c3-null transposon mutant HI2424Δc3.

one constructed using the entire genomes of the sequenced Bcc strains, indicating that the plasmid was acquired before clade divergence (Fig. S4). This provides strong evidence that a much smaller plasmid, carrying mainly the pC3 core genes, was originally present in a Bcc ancestor strain and then diverged by the accretion of foreign DNA into the different Bcc pC3 plasmids. This hypothesis is further supported by the phylogenetic analysis of Bcc *repA* and *parB* genes, which suggested that acquisition of the second and third replicons of Bcc genomes occurred before their differentiation into discrete species (Drevinek *et al.*, 2008a).

The vast majority of pC3 genes are not conserved between Bcc species (Fig. S5), but despite differing so greatly in gene content, the role of pC3 in virulence and antifungal activity is remarkably well-conserved, suggesting that the presence of the plasmid may increase the competitive fitness of the bacterium in its natural habitat. Plasmid pC3 has thus far only been identified in Bcc species, and it is therefore tempting to speculate that its presence is responsible for the intrinsic pathogenicity of strains belonging to this *Burkholderia* lineage.

The finding that pC3 is essential for pathogenicity in various hosts, but can be deleted, will open new avenues in the identification and analysis of virulence factors produced by Bcc strains. Moreover, the replication machinery of pC3 could serve as a novel target for the development of drugs that do not kill Bcc strains, but attenuate their virulence. Given the likelihood that pC3 plasmids from different strains can be exchanged and genetically modified, it may also be possible to design *Burkholderia* strains that are suitable for biotechnological applications.

## Experimental procedures

### *Ethics statement*

Animal experiments were conducted according to the guidelines of the Canadian Council of Animal Care for the care and use of experimental animals under protocol M08089 approved by the University of Calgary Animal Care Committee.

### *Bacterial strains and media*

All bacterial strains used in this study are shown in Table S4 (Casadaban and Cohen, 1980; Hanahan, 1983; Herrero *et al.*, 1990; Romling *et al.*, 1994; Hoang *et al.*, 1998). *E. coli* strains were routinely cultured at 37°C on LB Lennox agar containing appropriate antibiotics [Mueller–Hinton (DIFCO) was used when trimethoprim was used for selection]. Bcc strains were cultured at 37°C on LB lennox agar containing appropriate antibiotics, and maintained at room temperature on M9 minimal salts agar containing glucose (0.5% w/v) (Clowes and Hayes, 1968). Antibiotic concentrations used were as follows: chloramphenicol, 25 μg $ml^{-1}$ (*E. coli*), 50 μg $ml^{-1}$ (Bcc); trimethoprim, 25 μg $ml^{-1}$(*E. coli*), 50 μg $ml^{-1}$ (Bcc); gentamicin 20 μg $ml^{-1}$ (*E. coli* and Bcc).

M9 medium containing uracil as the nitrogen source was used for differentiation between wild-type and pC3-null Bcc strains. 210 ml 1.5% w/v agar (Conda) and glucose (0.5% w/v) was prepared in distilled water. This was autoclaved and allowed to cool to approximately 60°C. 30 ml × 10 M9 salts without any nitrogen source (60 g $Na_2HPO_4$, 30 g $KH_2PO_4$, 5 g NaCl, distilled $H_2O$ to a final volume of 1 l), was added, followed by 60 ml of a 0.33 % (w/v) uracil solution (filter sterilized). 300 µl each of filter sterilized 1 M $MgSO_4$ and 0.1 M $CaCl_2$ was added and the medium gently but thoroughly mixed.

### *Molecular methods*

All plasmids used in this study are shown in Table S4. Primers used in plasmid construction are shown in the Table S5. Plasmid DNA was routinely isolated using the Qiagen miniprep kit. DNA prepared by PCR amplification or restriction digestion was purified using the Qiagen PCR purification kit. General molecular methods were as described by Sambrook (Sambrook *et al*., 1989). Plasmids were introduced into Bcc strains by conjugation.

### *Conjugal transfer of plasmids*

Bacterial conjugations were carried out using a filter mating technique (Herrero *et al*., 1990), utilizing a helper strain (MM294/ pRK2013) to provide the *tra* genes. Conjugations were carried out on LB Lennox plates at 30–37°C for approximately 16 h. Ex-conjugants were selected on Pseudomonas Isolation Agar (DIFCO) containing appropriate antibiotics.

### *Construction of pMinic3*

The region of H111 pC3 containing the *parAB* and *repA* genes, two putative *parS* sites and two putative DnaA box motifs was identified *in silico*. This was predicted to be the region necessary for replication of this replicon. Approximately 900 bp of DNA bounding each end of this region was amplified and cloned into two suicide vectors (pSHAFT2 and pEX18Tp). In each case the reverse primer contained an FRT site. Primer sequences are shown in Table S5. The resultant vectors, pEX18TpMinupFrt and pSHAFT2MindownFrt, were inserted sequentially into H111 pC3, and correct integration was confirmed by PCR and sequencing. Integrants were Cm- and Tp-resistant. A plasmid bearing a flippase cassette (pBBR5::FLP), was introduced to stimulate recombination at the FRT sites. Colonies that had maintained Tp resistance, but lost Cm resistance, were isolated. These colonies were found to lack pC3, but contained instead the 12 644 bp pMinic3 plasmid.

The flippase-bearing plasmid, pBBR5::FLP was constructed as follows: the cassette containing the *flp* and *sacB* genes was excised from plasmid pFLP2 (Hoang *et al*., 1998) as a SacI/PstI fragment, and ligated into the broad host range plasmid vector pBBR1MCS-5 (Kovach *et al*. 1995). Ligation products were transformed into *E. coli* Top10 (Invitrogen) and transformants were selected on LB Lennox Gm 20 µg $ml^{-1}$.

### *Deletion of pC3 using pMinic3*

pMinic3 was purified from H111Δc3 and introduced into *E. coli* MC1061 by transformation. To improve the stability of pMinic3 in *E. coli*, bacteria were grown at 30°C. pMinic3 was introduced into Bcc strains by triparental conjugation. Following selection of ex-conjugants, colonies were purified and tested for the presence of pC3 by PCR using pC3-specific primers. Ten primer pairs were used, designed to anneal at evenly spaced intervals along the H111 pC3 (primer pairs 1-10Fc3 and 1-10Rc3, see Table S5). PCR screening of wild-type H111 with these primer pairs gives an ~ 1 kb fragment for each reaction. These fragments were not generated when pC3 was absent. When other Bcc members were used as the template for these PCR reactions, not all reactions resulted in specific products. However, in those reactions in which the wild-type template gave rise to a discrete fragment of approximately 1 kb, pC3-null candidates did not give rise to such a fragment.

pMinic3 was cured from pC3-deleted colonies by plating bacteria on M9 agar (Clowes and Hayes, 1968) containing 10% w/v sucrose as a carbon source. Trimethoprim sensitive colonies were tested for the absence of the pC3 replicatory region by PCR using primer pairs oriCFor1 and oriCRev1, and oriCFor2 and oriCrev2, with parent colonies used as a positive control. The absence of pC3 was confirmed by pulsed-field gel electrophoresis.

### *Pulsed-field gel electrophoresis (PFGE)*

Pulsed-field gel electrophoresis was carried out as described by Herschleb and colleagues (Herschleb *et al*., 2007). Agarose plugs were electrophoresed without restriction digestion to allow visualization of whole replicons. Settings for PFGE were as follows: 3.5 V $Cm^{-1}$, switch time 400–700 s, included angle 106°, run time 24 h. For *B. vietnamiensis* samples it was necessary to use different electrophoresis settings to prevent smearing. The settings used were as follows: 3.5 V $Cm^{-1}$, switch time 400–500 s, included angle 106°, run time 10 h. The buffer used for all PFGEs was 1× TAE.

### *Microarray analysis*

Cells were grown to $OD_{600}$ 2.5 in LB, and RNA was extracted using the RiboPure-Bacteria Kit (Ambion). Two-colour microarray experiments were performed at the School of Biosciences at Cardiff University using Agilent 4-pack Bcc gene chips (Holden *et al*., 2009). The chip comprises 10264 probes designed using *B. cenocepacia* sequences, consisting of 8741 J2315, 1070 AU1054 and 387 HI2424 probes. Three biological replicates of each strain were analysed. Total RNA (10–20 µg) was used for cDNA synthesis. First-strand cDNA was labelled using the CyScribe Post-Labelling Kit, and purified using the CyScribe GFX Purification Kit (GE Healthcare). cDNA was coupled with CyDye NHS ester, and labelled cDNA was purified using the CyScribe Post-Labelling Kit (GE Healthcare). Transcriptome analyses were performed in collaboration with the Cystic Fibrosis Foundation Therapeutics programme. Genespring (v.7.3.1) was used for data analysis, and data were further processed using the Affimetrix FE data normalization procedure recommended for Agilent arrays. Statistical analysis was carried out using ANOVA and the Benjamini and Hochberg False Discovery Rate (BH_FDR) multiple testing correction. Only differentially

expressed genes that hybridized to array-probes belonging to *B. cenocepacia* strain J2315 were included in this study. All microarray data have been deposited in the ArrayExpress database under accession number E-MTAB-732 (http://www.ebi.ac.uk/arrayexpress).

### *Phenotypic analyses*

*Phenotypic microarray.* Phenotypic microarrays were carried out using Biolog PM plates. PM1, 2a and 3b were used. PM 1–3 test the range of carbon and nitrogen sources that a bacterial strain is capable of utilizing. Assays were carried out following the protocol supplied by the manufacturer. Glycerol stocks of bacterial strains were grown on R2A agar, and passaged once on the same medium before testing. Cells were resuspended from R2A agar plates in Biolog inoculating fluid for analysis. Biolog microtitre plates were incubated at 30/37°C for 24 h. The $OD_{590}$ of each well was determined using a plate-reader (Biotek). The following criteria were used to determine whether a phenotype differed between the wild-type and pC3 deletion derivative strain: at least 50% difference between the $OD_{490}$ values for a given phenotype, with the higher value being at least 0.3. Where both wild type and Δc3 gave rise to $OD_{490}$ values > 1 for a given phenotype, no difference was scored. Each phenotypic difference was checked visually.

*Biofilm assay.* Biofilm assays were carried out in 96-suspension well microtitre plates (Sarstedt). Bacteria were cultured overnight in LB at 30°C, and 1 ml harvested by centrifugation and resuspended in fresh 0.85% (w/v) saline. Bacterial suspensions of $OD_{600}$ 0.01 in AB medium (Clark and Maaloe, 1967) with 10 mM sodium citrate as a carbon source were prepared, and used to inoculate the microtitre plate (100 μl per well). At least seven wells were inoculated with each strain, and 100 μl uninoculated AB medium was added to the remaining wells as controls. Plates were incubated in humid conditions at 30°C for 48 h. Following incubation, $OD_{550}$ was measured using a plate reader (Biotek) to give an indication of bacterial growth. The medium was discarded and 100 μl crystal violet solution added to each well. After 30 min at room temperature, the dye was discarded and the plate washed carefully but thoroughly with distilled water. The plate was then left open to dry, and any crystal violet dye attached to biofilm in the wells was dissolved with DMSO (120 μl per well). Biofilm was quantified by measuring absorbance at 570 nm.

*EPS production assay.* EPS production was visualized using mannitol agar plates [2 g yeast extract, 20 g Mannitol, 15 g agar (Conda), $H_2O$ to 1 l]. Bacteria were streaked on this medium in cross-shapes, taking care to use a similar amount of bacterial inoculum for each strain. Plates were incubated at 30°C for 3 days, and then at room temperature for two more days.

Protease production assay. Bacterial culture (5 μl, $OD_{600}$ 1) was spotted onto skimmed milk agar [2% (w/v) skimmed milk, LB broth Lennox (DIFCO), 1% agar (Conda)]. Plates were incubated overnight at 37°C. The assay was carried out in triplicate.

*Antifungal activity assay.* Fungus was prepared for this assay as follows: a fungal plug was transferred into the centre of a malt agar plate, sealed with parafilm and stored in the dark at room temperature until the fungal mycelium had reached the edge of the plate (~ 3 days for *Rhizoctonia solani*, and ~ 9 days for *Fusarium solani*). Bacteria were cultured overnight in LB, to give saturated cultures of similar density. Malt agar (DIFCO) plates with 2% agar were inoculated at three points around the edge with 20 μl bacterial culture. Plates were incubated at 30–37°C for 24 h (dependent upon the optimal temperature for growth of the strain in question). Fungal plugs were prepared from the outer (youngest) edge of the fungal mycelium, and transferred to the centres of the malt agar plates. Plates were sealed with parafilm and incubated at room temperature in the dark until the fungal mycelium had reached the edge of the plate, or a clear zone of inhibition was visible around the bacteria. The assay was carried out in triplicate for each strain tested.

*Determination of bacterial growth rate.* Strains to be tested were grown overnight in LB broth at 37°C with shaking. The overnight cultures were diluted to $OD_{600}$ 0.5, and 1 ml of each was used to inoculate 50 ml aliquots of LB broth. Growth was monitored by taking samples immediately following inoculation, and at 30 min intervals thereafter, and determining $OD_{600}$.

### *Pathogenicity assays*

Caenorhabditis elegans *killing assay.* The nematode killing assay was performed as described previously (Kothe *et al.*, 2003). Briefly, bacterial strains were cultured overnight in 5 ml LB broth at 30°C, and 100 μl spread per well on six-well plates containing nematode growth medium (NGM II). After 24 h incubation at 30°C, ~ 25 L4 larvae of *C. elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis, USA), which had been synchronized with hypochlorite, were transferred to each well. The plates were incubated at 20°C, and the living worms were scored at 24, 48 and 120 h using a Leica M165 FC binocular microscope at a magnification of × 50. Each experiment was carried out in triplicate, and as a negative control the food source strain *E. coli* OP50 was used. The pathogenicity of each strain was scored according to the following criteria: (i) visible unhealthiness of the nematodes (reduced locomotive capacity and swollen intestine) at day 2, (ii) percentage of living worms on day 2 of the experiment ≤ 70% original number, and (iii) the total number of living worms at day 5 (including progeny nematodes) ≤ 50. If one, two or three criteria were met, the pathogenicity score was determined as 1, 2 or 3, respectively, where 3 indicates a very pathogenic strain (Cardona *et al.*, 2005). Where none of the three criteria were met, a score of 0 was given, indicating a non-pathogenic strain.

Galleria mellonella *killing assay.* Infection of *G. mellonella* larvae was performed essentially as described previously (Jander *et al.*, 2000; Seed and Dennis, 2008; Uehlinger *et al.*, 2009). Briefly, *G. mellonella* in the final larval stage (purchased from Fischerei Brumann, Zürich) were stored at 15°C and used within 1 week. Bacterial cultures (grown overnight in LB

at 30°C) were diluted 1:100 in 30 ml LB broth and incubated with shaking at 30°C to $OD_{600}$ 0.4–0.7. The bacteria were harvested, and the pellets resuspended in 10 mM $MgSO_4$ (Merck). The $OD_{600}$ was adjusted to 0.025, corresponding to $\sim 8 \times 10^6$ cfu $ml^{-1}$. Injection of this number *B. anthina* LMG20983 cells was not pathogenic to *G. mellonella*, and therefore a 10 × higher concentration of this strain and its pC3-null derivative was used. 10 µl aliquots were injected into the *G. mellonella* larvae via the hindmost proleg using a 1 ml syringe (BD Plastipak) with a 27 G × 7/8" needle (Rose Gmbh). For the negative control larvae, 10 µl of $MgSO_4$ was injected. To avoid contamination, the injection area was disinfected before inoculation using a cotton swab soaked in ethanol. Ten randomly chosen larvae were used per strain tested, and each experiment was carried out in triplicate. The infected animals were incubated in Petri-dishes at 30°C in the dark. At 20, 24, 48 and 72 h p.i. the number of dead larvae was counted. Larvae were considered dead when they did not respond to physical manipulation.

*Zebrafish embryo infection assays.* Plasmid pIN29, conferring strong expression of DSRed, was introduced into HI2424 and HI2424Δc3 by electroporation as described (Vergunst *et al*., 2010). DSred-expressing *B. cenocepacia* HI2424 and HI2424Δc3 were then analysed for virulence in the zebrafish (*Danio rerio*) embryo model as described (Vergunst *et al*., 2010). Zebrafish were handled according to french national regulations for animal welfare. In three independent experiments, embryos were microinjected with 20–200 cfu. Embryos from a single injection experiment were randomized and used for the three different assays (bacterial enumeration, survival assays and real-time analysis). An estimation of bacterial cfu was obtained by disrupting and plating embryos at time points 0, 24 and 48 h p.i. ($n = 5$ embryos per time point), as described in Vergunst *et al*. (2010). For survival assays, embryos ($n = 20$ per experiment) were individually kept in 24-well plates and analysed at regular time intervals for mortality (scored by absence of heartbeat). For microscopy, a Leica DM IRB inverted microscope and a Nikon AZ100 were used. Conditions, filter sets and imaging methods were as described (Vergunst *et al*., 2010). For statistical analysis, ANOVA was used for kinetics assays, and the Breslow generalized Wilcoxon test was used to determine significance of survival plots.

*Alfalfa assay.* The alfalfa infection assay was carried out as previously described (Bernier *et al*., 2008).

*Rat chronic lung infection assay.* Male Sprague-Dawley rats (150–175 g; Charles River Canada) were tracheotomized under anaesthesia and inoculated with approximately $10^7$ cfu of the appropriate strain embedded in agar beads as previously described (Cash *et al*., 1979). At 7 days p.i., the lungs were harvested and analysed for bacterial counts or histopathological changes as previously described (Bernier *et al*., 2003). Infiltration of haematoxylin and eosin stained lung sections with inflammatory cells and exudates was quantified using Image Pro Plus Software (Media Cybernetics, Bethesda, MD, USA).

Burkholderia cenocepacia *H111 genome sequence.* The draft *B. cenocepacia* H111 genome nucleotide sequence has been deposited in the European Nucleotide Archive with the master WGS Accession No. CAFQ00000000. The final pC3 assembly has been assigned the Accession No. CAFQ01000058.

*pC3 alignments and phylogeny.* Completed pC3 nucleotide sequences from 10 different sources were downloaded from NCBI (Accession No. AM747722, CP000960, NC_008544, NC_007509, NC_008392, CP000616, NC_008062, and NC_010557). The sequences were aligned using the Mauve software (Darling *et al*., 2010). Predicted ORFs conserved among all sequences were extracted and aligned using the CLC Genomics workbench alignment tool. Forty-four nucleotide sequences producing good alignments were selected, and the curated alignments were concatenated. A phylogenetic tree of the Bcc pC3 was constructed in the CLC Genomics workbench based on the combined alignment using a maximum likelihood phylogeny algorithm and the GTR substitution model.

The amino acid sequences of each predicted CDS were downloaded and clustered into orthologous groups using the ORTHOMCL program (Li *et al*., 2003) with the following parameters: –e 1.0e-6 –v 1000 –b 1000. Orthologous genes conserved in all ten Bcc pC3 species were identified and mapped onto the H111 pC3 sequence using the DNAplotter software (Carver *et al*., 2008).

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# Appendix 2

**Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains by using multiple infection hosts**

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# Identification of Specific and Universal Virulence Factors in *Burkholderia cenocepacia* Strains by Using Multiple Infection Hosts▽†

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**Over the past few decades, strains of the *Burkholderia cepacia* complex have emerged as important pathogens for patients suffering from cystic fibrosis. Identification of virulence factors and assessment of the pathogenic potential of *Burkholderia* strains have increased the need for appropriate infection models. In previous studies, different infection hosts, including mammals, nematodes, insects, and plants, have been used. At present, however, the extent to which the virulence factors required to infect different hosts overlap is not known. The aim of this study was to analyze the roles of various virulence factors of two closely related *Burkholderia cenocepacia* strains, H111 and the epidemic strain K56-2, in a multihost pathogenesis system using four different model organisms, namely, *Caenorhabditis elegans*, *Galleria mellonella*, the alfalfa plant, and mice or rats. We demonstrate that most of the identified virulence factors are specific for one of the infection models, and only three factors were found to be essential for full pathogenicity in several hosts: mutants defective in (i) quorum sensing, (ii) siderophore production, and (iii) lipopolysaccharide biosynthesis were attenuated in at least three of the infection models and thus may represent promising targets for the development of novel anti-infectives.**

The *Burkholderia cepacia* complex (BCC) comprises a group of the following 17 formally named bacterial species: *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia*, *Burkholderia ubonensis*, *Burkholderia latens*, *Burkholderia diffusa*, *Burkholderia arboris*, *Burkholderia seminalis*, *Burkholderia metallica*, *Burkholderia lata*, and *Burkholderia contaminans* (46, 69, 70, 71). Strains of the BCC are ubiquitously distributed in nature and have been isolated from soil, water, the rhizosphere of plants, industrial settings, hospital environments, and infected humans. Some BCC strains have enormous biotechnological potential and have been used for bioremediation of recalcitrant xenobiotics, plant growth promotion, and biocontrol purposes. At the same time, however, BCC strains have emerged as problematic opportunistic pathogens in patients with cystic fibrosis (CF) and in immunocompromised individuals (12, 19, 44, 46). The clinical outcomes of BCC infections range from asymptomatic carriage to a fulminant and fatal pneumonia, the so-called cepacia syndrome (30). Apart from acquisition from the environment, patient-to-patient transmission and indirect nosocomial acquisition from contaminated surfaces have caused several outbreaks within and between regional CF centers (55). Although all BCC species have been isolated from both environmental and clinical sources, *B. cenocepacia* and *B. multivorans* are most commonly found in clinical samples (12, 44).

Members of the BCC not only are opportunistic pathogens of humans but also can cause infections in a diverse range of species, including animals, nematodes, and plants (59). This allowed the development of various infection models, using the mouse or rat, the nematode *Caenorhabditis elegans*, onions, or the alfalfa plant as an infection host. More recently, larvae of the wax moth *Galleria mellonella* have been used as infection hosts of BCC strains (58). These models have been employed to investigate the virulence of different BCC species as well as of mutants to understand the importance of specific genes in disease. These infection models have also been applied to studies of host response, gene therapy, antimicrobial delivery, and immunization for prevention of BCC lung disease (6, 32, 51).

Previous work has identified several virulence factors that may play a role in infections caused by BCC strains. Some isolates have been demonstrated to be capable of surviving within eukaryotic cells, such as respiratory epithelial cells, macrophages, and amoebae (7, 50, 56). Other virulence factors that have been identified by the use of different infection models include the quorum-sensing system (40), biofilm formation (14), iron-chelating siderophores (16), proteases (15), type III and IV secretion systems (20, 24, 67), melanin production (77), catalase (38), lipopolysaccharide (LPS) (41), cable pili and flagella (57, 68), surface exopolysaccharides (11), a *lysR* regulator (4), capsule (29), and intrinsic antimicrobial resistance

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(10). Recently, a phenylacetic acid catabolic pathway was shown to be required for full pathogenicity of *B. cenocepacia* in the *C. elegans* infection model (37).

At present, knowledge on the importance of these factors in different infection hosts is scarce. This study was initiated to identify both host-specific and conserved mechanisms of pathogenicity in *C. elegans*, *G. mellonella*, alfalfa, and murine infection models. We demonstrate that some virulence factors are important for pathogenicity in more than one infection model, while other factors were found to be host specific. *N*-Acyl homoserine lactone (AHL)-dependent quorum sensing (QS) was identified as a highly conserved regulatory mechanism for expression of pathogenic traits. Siderophore production and intact LPS were important for virulence in all animal models. However, we also identified several virulence factors that were required for pathogenesis in only one of the models.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in the present study are described in Table S1 in the supplemental material. All strains were grown aerobically in modified Luria-Bertani (LB) broth (1) (containing 4 g of NaCl/liter instead of 10 g of NaCl/liter) at 37°C. Solid medium contained 15 g of agar/liter (Conda, Madrid, Spain). Growth was determined spectrophotometrically by measurement of the optical density at 600 nm ($OD_{600}$).

***G. mellonella* killing assays.** Infection of *G. mellonella* larvae was performed as described previously (31, 58), with some modifications. Caterpillars in the final larval stage (Brumann, Zurich, Switzerland, or Hebeisen, Zurich, Switzerland) were stored in wooden shavings at 15°C and used within 2 to 3 weeks. Bacterial overnight cultures grown in LB broth were diluted 1:100 to 30 ml and cultivated to an $OD_{600}$ of 0.4 to 0.7. Cultures were centrifuged, pellets were resuspended in 10 mM $MgSO_4$ (E. Merck, Dietikon, Switzerland), and the $OD_{600}$ was adjusted to 0.125, corresponding to approximately $4 \times 10^7$ CFU/ml. Bacterial suspensions were supplemented with 100 μg/ml ampicillin to prevent contamination, as the *Burkholderia* strains used are intrinsically resistant to ampicillin. Cultures of strains containing plasmids pMLBAD*aiiA* and pBAH27 were supplemented with 100 μg/ml trimethoprim and 15 μg/ml gentamicin, respectively. As a control, *Escherichia coli* OP50 cells were injected without additives. A 1-ml syringe (BD Plastipak, Madrid, Spain) with a 27-gauge by 7/8-in. needle (Rose GmbH, Trier, Germany) was used to inject a 10-μl aliquot into *G. mellonella* via the hindmost proleg. The injection area was previously disinfected with a cotton swab soaked in ethanol. Ten to fourteen healthy, randomly chosen larvae were injected per strain and incubated in petri dishes at 30°C in the dark. To monitor killing of animals due to physical injury or infection by contaminating pathogens, larvae were injected with 10 μl $MgSO_4$ containing appropriate antibiotics. The number of dead larvae was scored 20, 24, 40, 48, and 72 h after infection. Dead larvae turned black as a result of melanization and did not respond to touch. Experiments with more than one dead larva in the $MgSO_4$ control group were not considered and were repeated. Data are mean values for at least three independent experiments.

**Nematode killing assays.** Nematode killing assays were performed essentially as described by Kothe et al. (35). Briefly, overnight cultures were adjusted to a density of about $1.3 \times 10^4$ to $1.5 \times 10^4$ CFU/ml, and 100 μl of suspension was plated on six-well plates containing nematode growth medium (NGM II) for slow killing assays. After 24 h of incubation at 37°C, a bacterial lawn was formed, and approximately 20 to 40 hypochlorite-synchronized L4 larvae of *C. elegans* Bristol N2 (obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis) were used to inoculate the plates. The actual number of worms was determined by using a Stemi SV6 microscope (Zeiss, Oberkochen, Germany) at a magnification of ×50. Plates were then incubated at 20°C and scored for live worms, with nematodes considered dead when they failed to respond to touch. The percentage of live worms and their morphological appearance were registered after 2 days. After 5 days, the total number of nematodes, including parental and progeny nematodes (if existing), was scored. All experiments were carried out at least three times, and *E. coli* OP50 was used as a negative control in the assays. The pathogenicity of strains was scored according to the following criteria: (i) sick appearance at day 2, including reduced locomotive capacity and the presence of a distended intestine; (ii) percentage of live worms of ≤50% after 2 days; and (iii) total number of worms of ≤100 after 5 days. For differentiating mild from severe infections, the presence of any one, two, or three of these criteria was scored as 1, 2, or 3 (as described previously [7, 71]). While a strain was considered pathogenic when at least one criterion was observed, a strain was described as nonpathogenic when no symptoms of disease were observed during the course of the infection experiment (pathogenicity score of 0).

**Alfalfa virulence assays.** Alfalfa seeds (var. 57Q77) were provided by Pioneer Hi-Bred International, Inc. (Johnston, IA). Alfalfa seeds were prepared as previously described (5). To disinfect the seeds and to accelerate germination, they were immersed in concentrated sulfuric acid (approximately 20 ml for 300 seeds) for 20 min and then washed with 500 ml of distilled water ($dH_2O$) four times. The seeds were covered with 60 ml of sterile $dH_2O$ in a 125-ml Erlenmeyer flask and incubated at 32°C with shaking for 6 to 8 h to encourage uniform imbibition and germination. The seeds were rinsed twice with 60 ml of sterile $dH_2O$ and incubated overnight in 60 ml of sterile $dH_2O$ at 32°C with shaking. The following day, the seedlings were placed in 24-well plates (Becton Dickinson) containing 2 ml of water agar (4). A total of 30 μl (normalized to an $OD_{600}$ of 0.3) of overnight culture grown in 5 ml LB at 37°C with shaking was used to surface inoculate three unwounded seedlings per well. The 24-well plates containing seedlings were sealed with Parafilm and incubated in a warm room (37°C) under a desk lamp producing artificial light. Controls included seedlings inoculated with 10 μl of 0.85% NaCl and untreated seedlings. The seedlings were visually monitored for disease symptoms, including yellow leaves, stunted roots, and brown necrotic regions, at 5 days postinfection. Each strain was tested with 10 to 12 seedlings per experiment. Data represent the means ± standard deviations for three assays.

**Animal studies.** Chronic infection experiments were performed using the rat agar bead infection model originally described by Cash et al. (9), modified for *B. cenocepacia* as previously described (61). Groups of 10 rats were infected intratracheally with H111 or K56-2 encased in agar beads. On day 7 postinfection, lungs were removed from three to five animals per group, homogenized, serially diluted, and plated on Trypticase soy agar to determine the number of bacteria persisting in the lung. The lungs from four or five additional animals per group were fixed in 10% formalin and examined for quantitative and qualitative histopathological changes.

**Detection of AidA.** For Western blotting, whole-cell proteins were separated in a 15% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). The membrane was probed with anti-AidA antibodies (27), and detection reactions were performed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma, Steinheim, Germany) according to the recommendations of the manufacturer (Roche, Mannheim, Germany).

## RESULTS AND DISCUSSION

**BCC strains exhibit differential virulence in different infection models.** Previous work demonstrated that the nematode *C. elegans* is a valuable host for studying the virulence of *Burkholderia* species (8). However, it is not known whether factors identified in the nematode model contribute to virulence in other infection hosts. As an initial step to address this issue, we investigated the virulence of different BCC strains in two nonmammalian infection hosts, namely, *C. elegans* and *G. mellonella* (Table 1). While in most cases the virulence levels were similar in both infection models, some strains were pathogenic for one but not the other host. For example, *B. anthina* LMG20983 effectively killed *C. elegans* but was virtually avirulent in the *G. mellonella* model. The opposite is true for *B. vietnamiensis* LMG10929. Differential virulence, albeit with less dramatic effects, was also observed with *B. stabilis* R6281, *B. multivorans* LMG18822, and *B. cepacia* R18194.

Interestingly, even three closely related *B. cenocepacia* strains, H111, K56-2, and J2315, showed marked differences in pathogenic potential in the two infection models. J2315 was less virulent than K56-2 in *G. mellonella* and *C. elegans* (data not shown), in spite of the fact that both strains belong to the epidemic ET-12 lineage. Strains of this lineage are transmissible and have resulted in high mortality in CF patients (47, 64).

TABLE 1. Pathogenicity of various BCC strains for *G. mellonella* and *C. elegans*

| Strain | % Dead *G. mellonella* larvae (mean ± SD) | | Pathogenicity score for *C. elegans*[b] | Reference |
|---|---|---|---|---|
| | 24 h p.i. | 48 h p.i. | | |
| *B. cenocepacia* H111 | 3 ± 5 | 100 ± 0 | 3 | 54 |
| *B. cenocepacia* H111-*aiiA* | 3 ± 6 | 7 ± 12 | 0 | 75 |
| *B. cenocepacia* K56-2 | 67 ± 29 | 100 ± 0 | 3 | 45 |
| *B. cenocepacia* J2315 | 0 ± 0 | 78 ± 29 | 1 | 45 |
| *B. cepacia* R18194 | 71 ± 30 | 100 ± 0 | 2 | 65 |
| *B. stabilis* R6281 | 6 ± 10 | 56 ± 11 | 0 | 25 |
| *B. dolosa* LMG21820 | 12 ± 14 | 9 ± 16 | 0 | 72 |
| *B. ambifaria* LMG17828 | 13 ± 6 | 100 ± 0 | 3 | 13 |
| *B. ambifaria* LMG17828-*aiiA* | 3 ± 6 | 10 ± 10 | 0 | 75 |
| *B. multivorans* LMG18822 | 0 ± 0 | 42 ± 7 | 0 | 25 |
| *B. vietnamiensis* LMG10929 | 14 ± 13 | 100 ± 0 | 0 | 13 |
| *B. vietnamiensis* LMG10929-*aiiA* | 0 ± 0 | 3 ± 5 | 0 | 75 |
| *B. pyrrocinia* LMG21822 | 59 ± 12 | 100 ± 0 | 3 | 13 |
| *B. anthina* LMG20983 | 9 ± 16 | 18 ± 16 | 3 | 13 |
| *E. coli* OP50[a] | 0 ± 0 | 3 ± 0 | 0 | 43 |

[a] *E. coli* OP50 was included as a control.
[b] According to references 8 and 75.

This is in contrast to the CF isolate H111, which did not cause any symptoms in the patient and could not be detected in sputum samples 5 years after it had been isolated (22). Strain K56-2 killed *G. mellonella* at a higher rate than H111 did; however, the opposite effect was observed in *C. elegans* (Fig. 1).

The rat agar bead model for studying chronic respiratory infections has been used extensively to study virulence properties of *B. cenocepacia* strains K56-2 (29, 62) and J2315 (5). Since the pathogenicity of K56-2 and H111 varied in the *C. elegans* and *G. mellonella* models, we assessed the virulence of these strains in the agar bead model. There was no difference in the ability of H111 versus that of K56-2 to cause persistent infection in the rat, as the recovery rates from infected lungs were 4.9 ± 0.45 log CFU per ml of lung homogenate and 4.65 ± 0.41 log CFU per ml at 7 days postinfection, respectively. There was a marked qualitative difference between the strains, however, in the lung histopathology observed in infected animals. K56-2-infected lungs had extensive inflammatory infiltrates with predominantly polymorphonuclear cells, whereas H111-infected lungs showed only slight signs of inflammation (Fig. 2). Infections with a 10-fold higher inoculum of H111, resulting in 6.4 ± 0.14 log CFU recovered per ml of lung homogenate at day 7 postinfection, did result in increased lung histopathology. Although the pathology was similar to that observed with K56-2, the inflammation remained restricted to the airways (data not shown). Taken together, these results suggest that the virulence of BCC strains in a particular infection model is strain dependent, likely due to the expression of specific virulence factors.

**The role of QS in virulence in different infection models.** Most *Burkholderia* species produce *N*-octanoylhomoserine lactone ($C_8$-HSL), which is synthesized by the AHL synthase CepI. As the cell density increases, $C_8$-HSL accumulates in the growth medium until a critical threshold concentration is attained. At this point, $C_8$-HSL binds to its cognate LuxR-type receptor protein, CepR, which in turn leads to the induction or repression of target genes (18, 62). Previous work has shown that CepIR homologues contribute to the virulence of *Burkholderia* species in the nematode *C. elegans* (35) and in murine species (63).

Using a quorum quenching approach, i.e., the heterologous expression of the *Bacillus* sp. strain 240B1 AHL lactonase AiiA, Wopperer et al. (75) demonstrated that nematode pathogenicity is AHL dependent in all investigated BCC strains. Intriguingly, expression of AiiA in three of the investigated strains abolished virulence not only in *C. elegans* but also in *G. mellonella* (Table 1). These results suggest that the expression of factors required for killing of wax moth larvae is AHL regulated and strengthen the view that QS in BCC strains is a major checkpoint for the control of pathogenicity.

To investigate the role of QS in pathogenesis of *B. cenoce-*

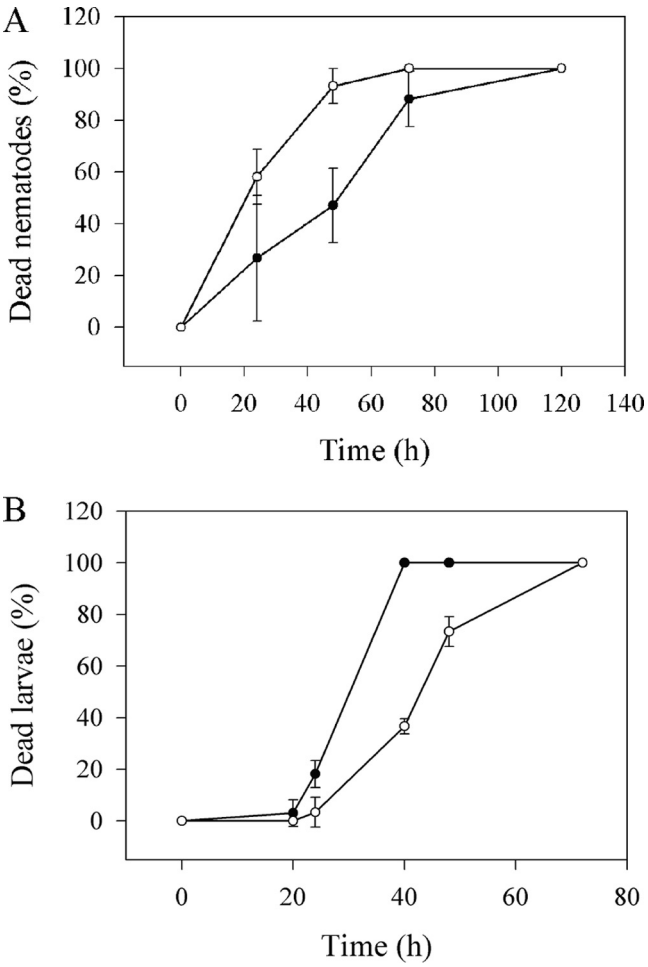


FIG. 1. Virulence of *B. cenocepacia* is strain specific. The killing kinetics of strains H111 (○) and K56-2 (●) were determined in the *C. elegans* (A) and *G. mellonella* (B) infection models. While strain H111 killed *C. elegans* faster than K56-2, the opposite was true for *G. mellonella*.

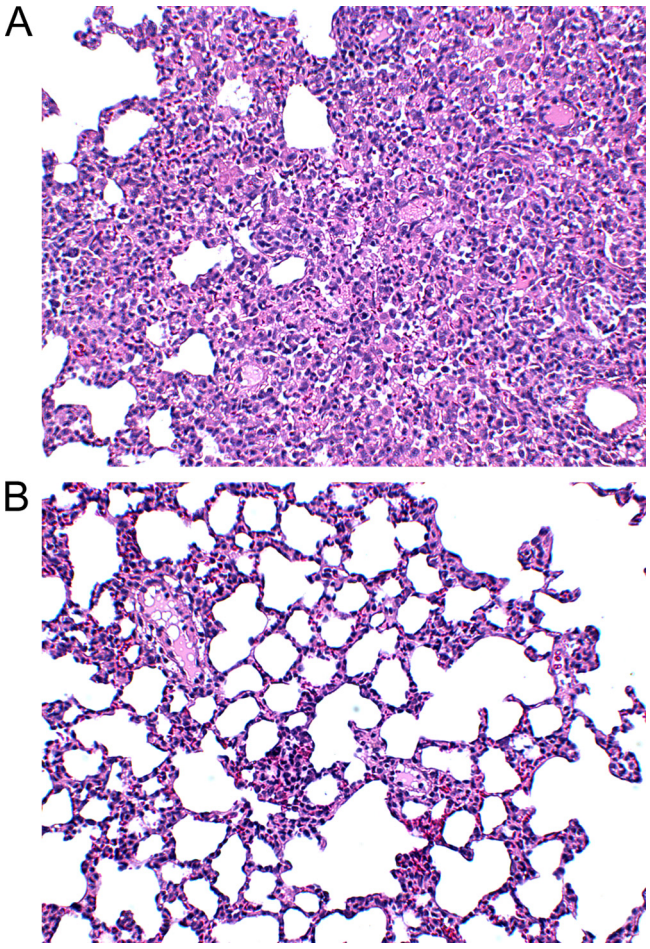


FIG. 2. *B. cenocepacia* strain K56-2 causes a greater inflammatory response in infected rat lungs than that induced by strain H111. The images show representative hematoxylin- and eosin-stained sections of rat lungs infected with *B. cenocepacia* K56-2 (A) and H111 (B). Mounted lung sections were examined by light microscopy for pathological changes, using an Olympus IX70 microscope, and images were taken using a cooled 12-bit charge-coupled device Retiga EXi camera (QImaging). Image analysis was performed using Volocity 4.2.0 software (Improvision Ltd.). Magnification, ×40. These sections were typical for groups of three animals.

*pacia* in better detail, the virulence of the wild type and of QS-deficient mutants of two *B. cenocepacia* strains, H111 and K56-2, was compared in *C. elegans*, *G. mellonella*, alfalfa, and a mouse or rat respiratory infection model (Table 2). In this context, it is important that the ET-12 strain K56-2 contains a second QS system, designated *cciIR*, which is present on the *c*enoc*e*pacia *i*sland (cci) and utilizes $C_6$-HSL as the major signal molecule (3, 48). Previous work demonstrated that *cciI* and *cepI* mutants are attenuated in a rat model of chronic lung infection (3). When *G. mellonella* was infected with $4 \times 10^5$ CFU of strain H111 or K56-2, the larvae died and turned black, as a result of melanization, within 48 h (Fig. 3). Inactivation of *cepI* in H111 reduced the virulence of this strain to the level of the control, indicating that QS plays an important role in pathogenesis of the wax moth. Surprisingly, however, we observed that the *cepR* mutant, H111-R, was as pathogenic as the wild type in *G. mellonella* (Table 2). This discrepancy may be explained by fact that CepR acts as both a positive and a negative regulator in *B. cenocepacia* (53). It is therefore likely that in the *cepR* mutant, expression of *G. mellonella*-specific virulence factors is derepressed. In contrast to the case with H111, neither the *cepI* mutant nor the *cciI* or *cepI cciI* mutant of K56-2 was attenuated in *G. mellonella*, indicating that the expression of virulence factors required for wax moth killing is AHL independent in K56-2.

Previous work has shown that both a *cepI* mutant and a *cepR* mutant of H111 exhibit attenuated virulence in *C. elegans* (35). In agreement with these results, we observed that the *cepI* mutant and the *cepI cciI* double mutant of K56-2, named K56-I2 and K56-2*cepIcciI*, respectively, showed reduced killing of *C. elegans* (Table 2). However, the *cciI* mutant, K56-2*cciI*, was found to be as virulent as the wild-type strain. Since one of the major virulence factors for *C. elegans* pathogenicity is AidA (27), we determined the expression levels of this protein in the wild-type and QS mutant strains. Western blot analysis using AidA-specific antibodies showed that expression of AidA is dependent on CepI but not on CciI (Fig. 4). The addition of AHLs to the growth media of the *cepI* and *cepI cciI* mutants restored not only expression of AidA but also virulence against *C. elegans* to the level of the wild type (data not shown). These results clearly demonstrate that AHL-dependent expression of AidA plays a central role in nematode pathogenicity of *B. cenocepacia*.

While infection of alfalfa with the *cepI* or *cepR* mutant of H111 showed less severe disease symptoms than those with the wild type, QS mutations had no effect on virulence of K56-2. These results suggest that either the QS circuitry controls only partially overlapping sets of genes or K56-2 has additional virulence factors that are expressed independent of AHL.

**Contributions of selected QS-regulated virulence factors to pathogenesis in different infection hosts.** To date, knowledge on the roles of QS-regulated virulence factors in different infection models is scarce. To address this issue in better detail, we tested mutant strains with defects in QS-regulated genes for virulence in different hosts.

In agreement with previous work (27), we found that the *aidA* mutant H111-A was greatly attenuated in *C. elegans* (Table 3). However, the mutant was as virulent as the wild-type strain in the wax moth, the alfalfa plant, and the lung infection model (Table 3; Fig. 2). These results indicate that AidA is not a general virulence factor but is highly specific for *C. elegans* pathogenicity.

The siderophore ornibactin, whose production is negatively QS regulated, has been demonstrated to be an important virulence factor in chronic respiratory infections (60, 61, 73). We tested two ornibactin-deficient mutants for virulence in the *C. elegans*, wax moth, and alfalfa infection models, including K56-2*orbA*, which is deficient in the outer membrane receptor for ornibactin (73), and K56-2I117, which is deficient in *pvdA*, a gene required for the production and uptake of ornibactin (61). Both mutants were found to be attenuated in virulence against *C. elegans* and *G. mellonella*. The *pvdA* mutant was slightly attenuated in the alfalfa model, but the *orbA* mutant was as virulent as the wild type. It is possible that ornibactin is taken up by other siderophore receptors, although less efficiently, which might explain the difference in attenuation between the *orbA* and *pvdA* mutants. Since the *pvdA* mutant was

TABLE 2. Virulence of QS mutants of *B. cenocepacia* H111 and K56-2 in different infection models[e]

| Strain | Function loss | % of wild-type virulence in *C. elegans*[a] | % of wild-type virulence in *G. mellonella*[b] | % Alfalfa seedlings with symptoms[c] | Result of animal infection study (mouse or rat)[d] | Reference(s) |
|---|---|---|---|---|---|---|
| H111-I | *cepI* | 23 ± 5* | 0 ± 0* | 17 ± 26* | NS | 25 |
| H111-R | *cepR* | 24 ± 12* | 100 ± 0 | 23 ± 32* | ND | 28 |
| H111-R (pBAH27) | *cepR* (*cepR*$^+$) | 100 ± 9 | 100 ± 0 | ND | ND | 28 |
| K56-I2 | *cepI* | 7 ± 4* | 100 ± 0 | 100 ± 0 | A, B | 40, 63 |
| K56-2*cciI* | *cciI* | 86 ± 12 | 100 ± 0 | 100 ± 0 | A | 3 |
| K56-2*cepIcciI* | *cepI cciI* | 0 ± 0* | 100 ± 0 | 100 ± 0 | ND | 48 |

[a] Since the killing rates of H111 and K56-2 in *C. elegans* differed greatly, the percentage of dead nematodes was determined after 48 h for H111 strains and after 72 h for K56-2 strains. Virulence of the wild-type strains was set to 100%.

[b] The percentage of dead larvae was determined at 48 h postinfection. Virulence of the wild-type strains was set to 100%.

[c] Inoculated alfalfa seedlings were inspected for disease symptoms, characterized by yellow or brown leaves and necrosis of the roots, at 5 days postinfection. Virulence of the wild-type strains was set to 100%. Assays were performed at least three times, with at least 10 seedlings per assay, and values shown are the means ± standard deviations for the three assays.

[d] Animal data for K56-2 strains are from references 2 and 61. The following pathogenicity indexes were used. An index of A indicates that lungs infected with mutants had 25 to 50% less inflammation than that with K56-2, determined by quantitative histopathology analysis of hematoxylin- and eosin-stained sections of lungs from chronic infections in the rat agar bead model. Lung sections were scanned using an Epson 1650 scanner, and areas of inflammation were digitized with Scion image software and reported as the percentage of lung inflammation. Means ± standard deviations for groups of four or five animals were significantly different ($P < 0.01$ by analysis of variance [ANOVA]). An index of B indicates that the strain was significantly less virulent than K56-2 in intranasally infected *Cftr*$^{-/-}$ mice, as determined by gross pathology scoring for inflammation; 2 to 3 log fewer bacteria were recovered from lungs ($P < 0.02$); and there was a lack of dissemination into the spleens of infected animals (61).

[e] Data are means ± standard deviations. *, significantly different from the wild type ($P < 0.05$ by ANOVA [alfalfa] or *t* test [*C. elegans* and *G. mellonella*]). ND, not determined; NS, no significant difference from the wild type.

only slightly attenuated, these results suggest that ornibactin-mediated iron uptake is an essential factor for pathogenicity in animals but not in plants.

The production of extracellular proteolytic activity is stringently QS regulated in BCC strains (23, 34). Two zinc metalloproteases, encoded by *zmpA* and *zmpB*, are positively regulated by CepR and CciR (34, 48, 63). Previous work has shown that inactivation of either *zmpA* or *zmpB* in K56-2 results in reduced histopathological changes in rats relative to those induced by the parent strain (15, 34). However, in *C. elegans*, *G. mellonella*, and alfalfa, no significant differences between mutant K56-2-9 (*zmpA*) or K56-2*zmpB*::tp (*zmpB*) and wild-type K56-2 could be observed (Table 3). Another protease, HtrA, which is localized in the periplasmic compartment and is expressed independent of AHL, was previously shown to be involved in osmotic and thermal stress resistance and required for persistence in the rat agar bead model of chronic lung infection (21). As with ZmpA and ZmpB, HtrA had no effect on pathogenicity of *B. cenocepacia* in the other infection models used in this study (Table 3). These data suggest that proteases are important for the infection of mammalian hosts only. One explanation for this finding could be that proteases are particularly important for modulating the host immune response by degrading specific tissue components, such as collagen and fibronectin, and for obstructing immune proteins, including neutrophil alpha-1 proteinase inhibitor, gamma interferon, and immunoglobulins (15, 33, 34). Since neither nematodes, insects, nor plants possess such advanced immune systems, the proteases may have no target and are therefore ineffective in these hosts.

We also tested a *gspE* mutant of H111, H111*gspE*, in *C. elegans*, *G. mellonella*, and alfalfa. H111*gspE* is mutated in the general secretory pathway, which is part of a type II secretion pathway responsible for the export of proteases and lipases (35) that has been shown to be negatively regulated by the CepR system in K56-2 (66). This mutant was not significantly attenuated in the three infection models (Table 3), suggesting that proteases and lipases are not important for virulence in plant and invertebrate hosts.

**Roles of mammalian virulence factors in pathogenesis of nonmammalian hosts.** Previous work identified several virulence factors that affect virulence in mammalian hosts, including *opcI*, a gene located on the cci island of ET-12 lineage strains which encodes an outer membrane protein that is closely related to the OmpC family of outer membrane bacterial porins and may act as a pore for the transport of small

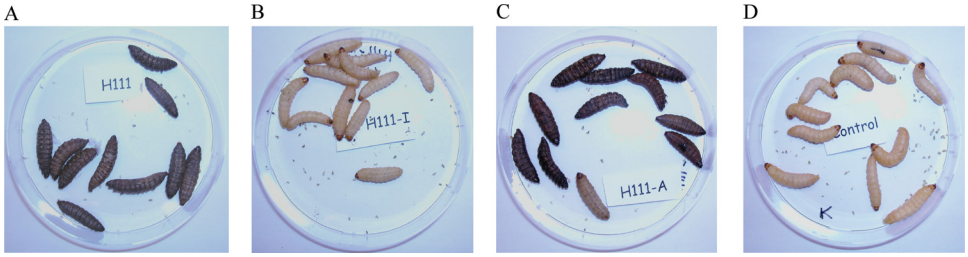


FIG. 3. CepI but not AidA is essential for *G. mellonella* pathogenicity of *B. cenocepacia* H111. Wax moth larvae were infected with $4 \times 10^5$ cells of the wild-type strain H111 (A), the *cepI* mutant H111-I (B), the *aidA* mutant H111-A (C), and the $MgSO_4$ control (D). Pictures were taken at 48 h postinfection.

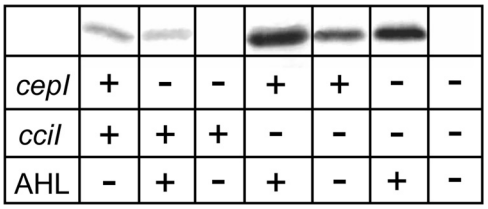


FIG. 4. Expression of AidA in *B. cenocepacia* K56-2 is CepI but not CciI dependent. Western blot analysis was performed with the wild-type strain K56-2, the *cepI* mutant K56-I2, the *cciI* mutant K56-2*cciI*, and the *cepI cciI* double mutant K56-2*cepIcciI*, using AidA-specific antibodies. The production of AidA is dependent on the presence of *cepI* but not *cciI*. The addition of external AHL molecules (100 nM of $C_8$-HSL for the *cepI* mutant, 100 nM of $C_6$-HSL for the *cciI* mutant, and both signal molecules for the double mutant) restored AidA expression of the *cepI* mutant to the level of the wild type.

molecules (3); *bscN*, which encodes a component of a type III secretion system (67); and *hldA*, which is involved in LPS biosynthesis (41).

The *opcI* mutant was attenuated only in the chronic respiratory infection rat agar bead model, not in any of the other models, suggesting a specific role of the encoded membrane protein for virulence in mammalian hosts (Table 3). The *bscN* mutant of K56-2 was found to be required for full virulence in *C. elegans* but not in *G. mellonella* or alfalfa. In agreement with the study of Markey et al. (49), we observed that the virulence of the *bscN* mutant was reduced 40% relative to that of the wild type, indicating that type III secretion is important not only for virulence in a murine model of infection (67) but also for nematode pathogenicity.

The SalI mutant is defective in expression of the *hldAD* operon, encoding two enzymes involved in the modification of heptose sugars prior to their incorporation into the LPS core oligosaccharide. The SalI mutant, which was previously shown to be defective in survival in the rat agar bead model of lung infection (41), was found to be attenuated in the *C. elegans* and *G. mellonella* infection models but was as virulent as the wild type in the alfalfa model. LPS is known to strongly stimulate the immune signal cascades in vertebrates and invertebrates via the innate immune system. The receptors responsible for the recognition of LPS belong to the family of Toll-like proteins (39). In this light, it may not be surprising that a mutant with an altered LPS structure is attenuated in pathogenicity. Interestingly, the mutant was not attenuated in alfalfa, in spite of the fact that plants are also capable of recognizing bacterial LPS and can trigger defense mechanisms in response (17). For example, the LPS of an endophytic *B. cepacia* strain was demonstrated to induce various defense genes in *Arabidopsis thaliana*, including a gene encoding an NO synthase. The observed difference in virulence in the animal and alfalfa models may be explained by the fact that the LPS receptor of the plant, which has no significant homology with the mammalian LPS receptor, has a much lower binding affinity for LPS (76).

The two shiny-colony variants K56-2 S76 and K56-2 0225 exhibit altered colony morphology as a result of a mutation of the BCAS0225 gene, which encodes a putative transcriptional regulator belonging to the LysR family (4). Both mutants were

TABLE 3. Virulence of various *B. cenocepacia* H111 and K56-2 mutants in different infection models[g]

| Strain | Mutation | % of wild-type virulence in *C. elegans*[a] | % of wild-type virulence in *G. mellonella*[b] | % Alfalfa seedlings with symptoms[c] | Result of animal infection study (mouse or rat)[d] | Reference |
|---|---|---|---|---|---|---|
| H111-A | *aidA* | 40 ± 19* | 94 ± 10 | 69 ± 53 | NS | 25 |
| H111*gspE* | *gspE* | 100 ± 6 | 83 ± 29 | 66 ± 32 | ND | 33 |
| K56-2*orbA*::tp | *orbA* | 66 ± 18* | 3 ± 6* | 98 ± 3[e] | A, B | 58 |
| K56-2-I117 | *pvdA* | 0 ± 0* | 41 ± 28* | 85 ± 13* | A, B | 59 |
| K56-2-9 | *zmpA* | 100 ± 11 | 100 ± 0 | 100 ± 0[e] | A, B | 14 |
| K56-2*zmpB*::tp | *zmpB* | 100 ± 17 | 100 ± 0 | 100 ± 0 | B | 32 |
| K56-2 RSF13 | *htrA* | 100 ± 13 | 100 ± 0 | 100 ± 0 | B | 20 |
| K56-2*bscN*::*cat* | *bscN* | 60 ± 24* | 100 ± 0 | 87 ± 15 | C | 67[f] |
| K56-2 SalI | *hldA* | 30 ± 37* | 52 ± 22* | 100 ± 0 | A | 39 |
| K56-2*opcI* | *opcI* | 97 ± 8 | 100 ± 0 | 100 ± 0 | B | 2 |
| K56-2 S76 | *lysR* | 100 ± 0 | 100 ± 0 | 0 ± 0[e]* | B | 3 |
| K56-2 0225 | *lysR* | 97 ± 5 | 100 ± 0 | 0 ± 0[e]* | ND | 3 |
| K56-2 RSF12 | Regulator | 99 ± 21 | 100 ± 0 | 96 ± 6 | A | 20 |

[a] Since the killing rates of H111 and K56-2 in *C. elegans* differed greatly, the percentage of dead nematodes was determined after 48 h for H111 strains and after 72 h for K56-2 strains. Virulence of the wild-type strains was set to 100%.

[b] The percentage of dead larvae was determined at 48 h postinfection. Virulence of the wild-type strains was set to 100%.

[c] Inoculated alfalfa seedlings were inspected for disease symptoms, characterized by yellow or brown leaves and necrosis of the roots, at 5 days postinfection. Virulence of the wild-type strains was set to 100%. Assays were performed at least three times, with at least 10 seedlings per assay, and values shown are the means ± standard deviations for the three assays.

[d] Data for K56-2 strains were taken from the literature. The following pathogenicity indexes were used. An index of A indicates that the mutant had a significantly reduced ability to persist at a level of at least 3 log in lungs of rats infected using the agar bead model ($P < 0.05$), as determined between 7 and 28 days postinfection. An index of B indicates that lungs infected with mutants had at least 50% less inflammation than those infected with K56-2, determined as described in Table 2. Means ± standard deviations for groups of four or five animals were significantly different ($P < 0.05$ by ANOVA). An index of C (from the results of Tomich et al. [67] comparing strain J2315*bscN*::*cat* to J2315 in the mouse agar bead model) indicates that the mutant had a significantly reduced ability to persist at a level of at least 3 log in lungs of infected mice at 72 h postinfection ($P < 0.02$ by Student's unpaired *t* test). Qualitative differences in lung histopathology were also reported between mutant and wild-type strains. ND, not determined; NS, not significantly different from the wild type when animals were infected with either $10^4$ or $10^5$ CFU of bacteria encased in agar beads.

[e] Data were taken from references 4 and 5.

[f] Tomich et al. (67) tested strain J2315*bscN*::*cat*.

[g] *, significantly different from the wild type ($P < 0.05$ by ANOVA [alfalfa] or *t* test [*C. elegans* and *G. mellonella*]).

avirulent in an alfalfa seedling infection model, and the S76 mutant also produced significantly less lung histopathology than the rough parental strain K56-2. However, both mutants were indistinguishable from the wild-type strain in their virulence in the *C. elegans* and *G. mellonella* infection models (Table 3). This indicates that BCAS0225 controls the expression of a factor(s) required for virulence in alfalfa and rats but not in *C. elegans* and *G. mellonella*.

K56-2 RSF12, a mutant with a defect in the response regulator BCAL2831, was shown to be unable to compete with the parental strain K56-2 in a rat model of chronic lung infection (21). However, this mutant was not attenuated in any other infection model used in this study.

**Conclusions.** Evidence that has accumulated over the past few years has demonstrated that there is a high degree of conservation of virulence mechanisms required to infect different hosts (36, 42, 74). It is thought that commonalities in bacterial virulence strategies exist because they initially evolved to combat their natural predators in the environment. This idea is also strongly supported by the fact that the innate immune systems of vertebrates and invertebrates share many common features that have been conserved during evolution (26). Given the practical advantages in their use, nonmammalian infection models have been established as attractive alternatives to traditional animal models (36).

Strains of the BCC are commonly found in the rhizosphere of plants (52), and it is likely that these organisms have evolved defense mechanisms that confer on them a survival advantage in this niche. In fact, BCC strains have been shown to infect various hosts, including mammals, nematodes, and plants (for a review, see reference 59). Depending on the infection model, various factors have been implicated in virulence in these models (62). In this study, we investigated to what extent the virulence factors required for pathogenesis in the different hosts overlap. In agreement with previous studies, we show that AHL-mediated QS plays a central role in the control of pathogenic traits in BCC strains. Rather than controlling one universal pathogenicity factor, our data support a model in which QS regulates the expression of a battery of virulence factors, which vary in their importance for infections in different host organisms. For example, AidA, whose expression is stringently QS regulated and which is essential for killing of *C. elegans*, played no role in any of the other pathogenesis models and thus appears to be a specific virulence factor required for infection of nematodes. AHL-dependent production of proteases was found to be important for pathogenicity of *B. cenocepacia* in mammals but not in invertebrates or alfalfa. QS-regulated synthesis of siderophores is important for virulence in mammals, *C. elegans*, and *G. mellonella* but not in alfalfa. Since the expression of virulence factors in multiple hosts is coordinated by AHL-dependent QS systems, we believe that QS may be a particularly attractive target for the development of compounds that can be used for treating BCC strain infections.

Interestingly, QS mutants of K56-2, although attenuated in a mammalian model and the *C. elegans* infection model, were as virulent as the wild type in the alfalfa and *G. mellonella* models, suggesting that in this strain the expression of some virulence factors is AHL independent. Furthermore, our investigations identified several virulence factors which, to our knowledge, are expressed independent of AHL and are important for pathogenicity in some of the infection models but not others. In this context, it is worth noting that the production of LPS was found to be particularly critical for infection, as a mutant with a defective LPS structure was attenuated in all animal models.

These studies also demonstrate that several virulence factors are host specific, with little correlation between seemingly related invertebrate models. BCC strains appear to utilize different virulence mechanisms to compete for survival in soil nematodes, plants, and mammalian hosts. This strategy is markedly different from the one employed by the opportunistic pathogen *Pseudomonas aeruginosa*, which was demonstrated to utilize virulence mechanisms that are conserved in diverse infection hosts (1, 36, 42). In fact, in *P. aeruginosa* only a few host-specific virulence factors could be identified, and the large overlap between virulence factors required for nonmammalian and mammalian pathogenesis validated the use of invertebrates as surrogate hosts. We identified only a few universal virulence factors in *B. cenocepacia*, and we therefore believe that extrapolations from nonmammalian infection models to mammalian infections must be used with caution.

## ACKNOWLEDGMENTS

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# Appendix 3

**LasI/R and RhlI/R quorum sensing in a strain of *Pseudomonas aeruginosa* beneficial to plants**

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# LasI/R and RhlI/R Quorum Sensing in a Strain of *Pseudomonas aeruginosa* Beneficial to Plants[▽]

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***Pseudomonas aeruginosa*** **possesses three quorum-sensing (QS) systems which are key in the expression of a large number of genes, including many virulence factors. Most studies of QS in *P. aeruginosa* have been performed in clinical isolates and have therefore focused on its role in pathogenicity. *P. aeruginosa*, however, is regarded as a ubiquitous organism capable of colonizing many different environments and also of establishing beneficial associations with plants. In this study we examined the role of the two *N*-acyl homoserine lactone systems known as RhlI/R and LasI/R in the environmental rice rhizosphere isolate *P. aeruginosa* PUPa3. Both the Rhl and Las systems are involved in the regulation of plant growth-promoting traits. The environmental *P. aeruginosa* PUPa3 is pathogenic in two nonmammalian infection models, and only the double *las rhl* mutants are attenuated for virulence. In fact it was established that the two QS systems are not hierarchically organized and that they are both important for the colonization of the rice rhizosphere. This is an in-depth genetic and molecular study of QS in an environmental *P. aeruginosa* strain and highlights several differences with QS regulation in the clinical isolate PAO1.**

*Pseudomonas aeruginosa* has been intensively studied by the scientific community because it is an opportunistic pathogen able to chronically colonize and infect cystic fibrosis patients (30). An important aspect of this bacterium is its capability to adapt to the host environment through the extensive and complex transcriptional regulation of an arsenal of virulence genes. A key player in this response is the quorum-sensing (QS) cell-cell communication system, which coordinates the behavior of *P. aeruginosa* communities. In fact, the transcriptional regulation of many virulence genes is controlled by two *N*-acyl homoserine lactone (AHL)-dependent QS systems called LasI/R and RhlI/R (16, 48).

In the LasI/R system, *lasI* directs the synthesis of *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL), which binds and activates the cognate response regulator LasR, resulting in the regulation of target gene expression. In the RhlI/R system, on the other hand, *rhlI* directs the synthesis of *N*-(butanoyl)-homoserine lactone (C4-HSL), which then interacts with the cognate RhlR, influencing transcription of target genes. These two QS systems are probably among the most studied in bacteria, and their regulons are fundamental to the pathogenicity of *P. aeruginosa* (48). Importantly, the two systems are intimately connected, being hierarchically organized with the LasI/R system regulating the transcription of *rhlI-rhlR* (29). The two QS regulons overlap, and together they constitute approximately 10% of the genes in *P. aeruginosa*, including factors like elastase, alkaline protease, exotoxin A, rhamnolipids, and pyocyanin, as well as being important for the regulation of biofilm formation (19, 46, 58). The two systems are themselves controlled by different regulators, allowing QS to respond and be modulated also by an array of environmental stimuli (56).

The importance of QS in the pathogenicity of *P. aeruginosa* has been demonstrated using a number of models, including insect, animal, and worm. The sputum of cystic fibrosis patients has been found to contain AHL molecules, virulence factors, and *lasI-lasR* transcripts, indicating that QS is active in vivo (14). The large majority of studies on AHL-dependent QS systems in *P. aeruginosa* have used the model strain PAO1, which was isolated from an infected wound over 50 years ago (54). In spite of the fact that *P. aeruginosa* is considered to be a ubiquitously distributed bacterium and is able to competitively colonize several environments, including soil, marshes, marine habitats, and plant roots, environmental strains have been hardly investigated (7). In this study we analyzed the LasI/R and RhlI/R QS systems of *P. aeruginosa* strain PUPa3 (28), a plant growth-promoting rice rhizosphere isolate which exhibits a wide range of antifungal activities and other beneficial traits related to the plant-bacteria interaction. It is shown that this strain harbors two QS systems that are highly homologous to the LasI/R and RhlI/R systems of PAO1. However, in contrast to PAO1, the two systems of PUPa3 are not hierarchically arranged. The roles of these two systems in root colonization, virulence toward *Caenorhabditis elegans* and the wax moth *Galleria melonella*, and for expression of several other phenotypic traits have been investigated.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and recombinant DNA techniques.** *P. aeruginosa* (strain PUPa3), which has strong antifungal activity and several plant

TABLE 1. Bacterial strains, plasmids, and primers used in this study

| Strain, plasmid, or primer | Relevant characteristics or sequence | Reference or source |
|---|---|---|
| *E. coli* strains | | |
| DH5α | F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* (*lacZYA-argF*)*U169 deoR* [80d*lac*(*lacZ*)M15*recA1*] | 44 |
| JM109 | *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA* [F′ *traD36 proAB lacI*$^q$ *lacZ*ΔM15 | 63 |
| *C. violaceum* CV026 | Double mini-Tn*5* mutant from *C. violaceum* ATCC 31532, AHL biosensor | 36 |
| *P. putida* F117 | AHL-negative derivative of *P. putida* IsoF; PpuI$^-$ | 50 |
| *P. aeruginosa* strains | | |
| PAO1 | Wild type | 21 |
| PAO1*lasI*::Gm | PAO1 with Gm cartridge inserted into unique EcoRI site of *lasI* | 3 |
| PAO1*rhlI*::Tc | PAO1 with Tc cartridge inserted into unique EcoRI site of *rhlI* | 3 |
| PAO1*rsaL* | *rsaL*::IS*lacZ-hah* | 42 |
| PUPa3 | Wild type, rice rhizosphere isolate | 28 |
| LASI | *lasI*::Km of *P. aeruginosa* PUPa3; Km$^r$ | This study |
| LASR | *lasR*::Km of *P. aeruginosa* PUPa3; Km$^r$ | This study |
| RHLI | *rhlI*::Gm of *P. aeruginosa* PUPa3; Gm$^r$ | This study |
| DMI | *lasI*::Km *rhlI*::Gm of *P. aeruginosa* PUPa3; Km$^r$ Gm$^r$ | This study |
| DMR | *lasR*::Km *rhlR*::Tet of *P. aeruginosa* PUPa3; Km$^r$ Tet$^r$ | This study |
| RSAL | *rsaL*::Tc of *P. aeruginosa* PUPa3; Tet$^r$ | This study |
| Plasmids | | |
| pKRC12 | pBBR1MCS-5 carrying $P_{lasB}$-*gfp* (ASV) $P_{lac}$-*lasR*; Gm$^r$ | 43 |
| pSB1075 | *lasR*-$P_{lasI}$ *luxCDABE*; Amp$^r$ | 61 |
| pRK2013 | Tra$^+$ Mob$^+$ ColE1 replicon; Km$^r$ | 15 |
| pMOSBlue | Cloning vector; Amp$^r$ | Amersham-Pharmacia |
| pBluescript KS | Cloning vector; Amp$^r$ | Stratagene |
| pLAFR3 | Broad-host-range cloning vector, IncP1; Tet$^r$ | 49 |
| pIB101 | pLAFR3 containing *P. aeruginosa* PUPa3 DNA | This study |
| pIB103 | pLAFR3 containing *P. aeruginosa* PUPa3 DNA | This study |
| pMULTIAHLPROM | Broad-host-range plasmid containing eight *luxI*-type promoters fused to a promoterless *lacZ* gene; Tet$^r$ | 52 |
| pKNOCK-Km | Conjugative suicide vector; Km$^r$ | 1 |
| pKNOCK-Gm | Conjugative suicide vector; Gm$^r$ | 1 |
| pKNOCK-Tet | Conjugative suicide vector; Tet$^r$ | 1 |
| pKNOCK-lasI | Internal PCR fragment of *P. aeruginosa* PUPa3 *lasI* cloned in pKNOCK-Km | This study |
| pKNOCK-rhlI | Internal PCR fragment of *P. aeruginosa* PUPa3 *rhlI* cloned in pKNOCK-Gm | This study |
| pKNOCK-rhlR | Internal PCR fragment of *P. aeruginosa* PUPa3 *rhlR* cloned in pKNOCK-Tet | This study |
| pKNOCK-rsaL | Internal PCR fragment of *P. aeruginosa* PUPa3 *rsaL* cloned in pKNOCK-Tet | This study |
| Primers | | |
| lasI-for | GAAATCGATGGTTATGACGC | This study |
| lasI-rev | CGGCACGGATCATCATCTTC | This study |
| rhlI-for | TCAGGTCTTCATCGAGAAGC | This study |
| rhlI-rev | CGTTGCGAACGAAATAGCG | This study |
| rhlR-for | TGGATCCGGCGATCCTCAAC | This study |
| rhlR-rev | GCTCTAGAGCTTCTGGGTCAGCAACT | This study |
| rsaL-for | TTGGATCCACCCGCACCGCCCGAC | This study |
| rsaL-rev | GCTCTAGATATATAGGGAAGGGCAGG | This study |

growth-promoting traits, has been previously isolated from the rice rhizosphere in India (28). This strain was grown at 28°C in either Luria-Bertani (LB) agar, M9 minimal medium (44), or King medium (24). Strains and plasmids used in this study are listed in Table 1. AHL bacterial biosensors used for AHL detection were *Chromobacterium violaceum* strain CVO26, *Escherichia coli* JM109(pSB1075), and *Pseudomonas putida* F117(pKRC12) (for all, see reference 53). For DNA transformations DH5α (18) was used while for triparental matings we used *E. coli* DH5α(pRK2013) (15). Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg ml$^{-1}$; tetracycline, 15 μg ml$^{-1}$ (*E. coli*) or 100 μg ml$^{-1}$ (*P. aeruginosa*); gentamicin, 10 μg ml$^{-1}$ (*E. coli*) or 100 μg ml$^{-1}$ (*P. aeruginosa*); kanamycin, 50 μg ml$^{-1}$ (*E. coli* and *C. violaceum*) or 300 μg ml$^{-1}$ (*P. aeruginosa*).

Recombinant DNA techniques, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, end filling with Klenow enzyme, hybridization, radioactive labeling by random priming, and transformation of *E. coli* were performed as described previously (44). Southern hybridizations were performed using N$^+$ Hybond membranes (Amersham, Biosciences); plasmids were purified using Jet Star columns (Genomed GmbH, Löhne, Germany) or the alkaline lysis method (6); total DNA from *Pseudomonas* was isolated by Sarkosyl-pronase lysis as described previously (5).

**Extraction and visualization of AHL signal molecules.** For the detection of the AHL molecules, *P. aeruginosa* strain PUPa3 was grown overnight in 20 ml of M9 minimal medium supplemented with glucose and Casamino Acids, and the supernatant of the culture was extracted with an equal volume of ethyl acetate acidified with 0.1% acetic acid. The preparation was centrifuged (5,000 rpm for 5 min) and the ethyl acetate phase collected. The extract was then dried and resuspended in a small volume of ethyl acetate and was run on $C_{18}$ reverse-phase chromatography plates, with synthetic AHLs used as standards (which were purchased either from Fluka-Sigma-Aldrich or from P. Williams, University of Nottingham, United Kingdom), using 60% (vol/vol) methanol in water as the

mobile phase. The plates were overlaid with a thin layer of LB agar seeded with either *C. violaceum* CVO26 or *E. coli*(pSB1075).

**Cloning and inactivation of QS genes in *P. aeruginosa* PUPa3.** A cosmid library was constructed for *P. aeruginosa* PUPa3 using the cosmid pLAFR3 (49) as vector. Insert DNA was prepared by partial EcoRI digestion of the chromosomal DNA of strain PUPa3 and then ligated in the corresponding site of pLAFR3. The ligated DNA was then packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene), and the phage particles were transduced to *E. coli* HB101. In order to identify the cosmid containing the QS genes, the *E. coli* HB101 harboring the cosmid library was used as donor in a triparental conjugation with, as acceptors, the AHL biosensor *C. violaceum* CV026 or *P. putida* F117(pKRC12). Transconjugants in which the biosensor was activated (i.e., restoration of purple pigment or green fluorescent protein expression) were considered positive and were extracted. Cosmid pIB103 harbored the *lasI-lasR* genes of *P. aeruginosa* PUPa3; part of a ClaI-SacI fragment (approximately 5.5 kb) was sequenced and found to include a partial QS locus. To obtain the remaining part of the *lasI* gene, a primer walking procedure on the original cosmid was performed. Cosmid pIB101 harbored the *rhlI-rhlR* genes of *P. aeruginosa* PUPa3; two adjacent ClaI-SacI fragments (approximately 4.5 and 0.6 kb) were cloned into pBSIIKS and their sequences were found to include the *rhlI-rhlR* genes. The QS genomic null mutants were created via insertional mutagenesis utilizing the conjugative suicide vectors pKNOCK-Km, pKNOCK-Gm, and pKNOCK-Tc (1). For the *lasI* (3-oxo-C12-HSL synthase gene) mutant, part of this gene (343 bp) was amplified using oligonucleotides lasI-for and lasI-rev and cloned as an XbaI-BamHI fragment in pKNOCK-Km to yield pKNOCK-lasI. This latter plasmid was then used as a suicide delivery system in order to create a *lasI* knockout mutant through homologous recombination, generating LASI. Similarly, the *rhlI* (C4-HSL synthase gene) mutant was created by amplifying part of this gene (377 bp) using oligonucleotides rhlI-for and rhlI-rev and cloning it as a XbaI-BamHI fragment into pKNOCK-Gm to yield pKNOCK-rhlI, which was used to generate RHLI. The double mutant *lasI rhlI* was obtained by conjugating pKNOCK-rhlI into *lasI* mutant LASI, generating DMI (double mutant I). To generate the *lasR* (transcriptional regulator) mutant (LASR), first cosmid pIB103 was mutagenized using transposon Tn*5* (as described in reference 32) with *E. coli* HB101::Tn*5* as source of the transposon. Thereafter, the cosmids containing Tn*5* were selected by conjugating them in strain *P. putida* WCS358, extracting, and transforming them in *E. coli* DH5α in order to conjugate them into the biosensor *P. putida*(pKRC12). Colonies that did not express the reporter gene were selected, and the position of Tn*5* in the *lasR* gene was confirmed through arbitrary PCR as previously described (40) and sequencing. The cosmid harboring the Tn*5* (pIB103::Tn*5*) carrying a Tn*5* insertion in the *lasR* gene was homogenized with the corresponding target region of the genome of *P. aeruginosa* PUPa3 by a marker exchange procedure (8, 57). pPH1JI was used as the incoming IncP1-incompatible plasmid. The *lasR rhlR* (transcriptional regulators) double mutant (DMR) was created by amplifying part of the *rhlR* gene (266 bp) using oligonucleotides rhlR-for and rhlR-rev and cloning it as a BamHI- XbaI fragment into pKNOCK-Tc to yield pKNOCK-rhlR, and the latter was then conjugated into LASR to generate DMR (*lasR rhlR* double mutant). Finally, the *rsaL* (negative transcriptional regulator) mutant was created by amplifying part of the *rsaL* gene (126 bp) using oligonucleotides rsaL-for and rsaL-rev and cloning it as a BamHI- XbaI fragment into pKNOCK-Tc to yield pKNOCK-rsaL, and the latter was then conjugated into the *P. aeruginosa* wild-type strain to obtain the mutant RSAL. The fidelities of all marker exchange events were confirmed by Southern analysis (data not shown). All mutants were tested for their growth rate in LB and M9 minimal media and showed a behavior similar to the wild-type strain (data not shown).

**In vivo models of infection. (i) Root colonization assay.** Root colonization assays were performed with the following strains: the wild-type *P. aeruginosa* PUPa3 (PUPA), the *lasI* derivative mutant (LASI), the *lasR* derivative mutant (LASR), the *rhlI* derivative mutant (RHLI), the double derivative mutant *lasI rhlI* (DMI), the double derivative mutant *lasR rhlR* (DMR), and the *rsaL* mutant derivative (RSAL).

Rice seeds (cultivar BALDO; kindly provided by the Ente Risi, Pavia, Italy) were first sterilized by soaking them in 5% sodium hypochlorite for 30 min and then washed six times (3 min for each wash) in sterile water. Then, under sterile conditions, each seed was placed in a 50-ml test tube containing 7.5 ml of half-diluted Hoagland solution (20) and placed at 30°C for germination. Six days later the germinated seedlings were treated with bacterial suspensions ($10^8$ CFU $ml^{-1}$) for 10 min. The seedlings were subsequently transferred to new 50-ml test tubes containing sterile perlite and then filled with half-diluted Hoagland solution containing $10^8$ CFU of *P. aeruginosa* PUPa3 inoculant for every gram of perlite present. After 1 day in a 30°C growth chamber the plants were transferred to a full-containment greenhouse for an additional 10 days. In the greenhouse natural daylight was supplemented with 500-W lamps with one lamp for 2 square meters and a 16-h/8-h light-dark cycle. The temperature was kept at 27°C and humidity at 70%. One day after the transfer to the greenhouse, a hole was made on the bottom of each test tube to allow drainage. From that moment onwards, 15 ml of sterile half-diluted Hoagland solution was added to each plant every other day. Eleven days after bacterial treatments, roots were harvested, separated from perlite, weighed, and finally ground in 5 ml physiological solution (0.85% NaCl). Serial dilutions were plated (on LB ampicillin), and CFU were counted on the following day after overnight incubation at 30°C. Values of root colonization are given as CFU $g^{-1}$ of root. Four separate root colonization assays were performed. In the first experiment the strains tested were as follows: (i) PUPa3 wild-type strain, (ii) LASI (*lasI* mutant), and (iii) LASR (*lasR* mutant). In the second independent experiment the strains tested were (i) PUPa3, (ii) LASI (*lasI* mutant), and (iii) DMI (*lasI rhlI* double mutant). In the third experiment we tested (i) PUPa3, (ii) DMR (*lasR rhlR* double mutant), and (iii) RSAL (*rsaL* mutant). Finally in the fourth experiment we tested (i) PUPa3, (ii) LASI (*lasI* mutant), (iii) RHLI (*rhlI* mutant), (iv) DMI (*lasI rhlI* double mutant), (v) DMR (*lasR rhlR* double mutant), and (vi) RSAL (*rsaL* mutant). In the first two experiments we used 8 plant replicates per treatment, in the third experiment we used 8 plant replicates for the wild-type strain and 24 plants for each of the mutants, and in the fourth experiment we used 10 plant replicates for each bacterial treatment. The statistical significance of the differences between the wild type and mutant strains in root colonization ability was tested with an analysis of variance (ANOVA; with wild-type PUPa3, LASI [*lasI*], RHLI [*rhlI*], DMI [*lasI rhlI*], DMR [*lasR rhlR*], and RSAL [*rsaL*] as independent variables) and then the specific differences were tested using planned comparisons.

**(ii) *Caenorhabditis elegans* and *Galeria melonella* killing assays.** Infection of *G. mellonella* larvae was performed as described previously (23, 47) with some modifications. Caterpillars in the final larval stage (Brumann or Hebeisen, Zürich, Switzerland) were stored in wooden shavings at 15°C and were used within 2 to 3 weeks. Bacterial overnight cultures grown in LB broth at 37°C were diluted 1:100 in 30 ml LB and grown to an optical density at 600 nm ($OD_{600}$) of 0.3 to 0.9. Cultures were centrifuged, and pellets were resuspended in 10 mM $MgSO_4$ (E. Merck, Dietikon, Switzerland), and the $OD_{600}$ was adjusted to 1.0. A 10-μl aliquot of a $10^{-5}$ dilution of this suspension, corresponding to approximately 30 CFU/ml, was used to infect the larvae using a 1 ml syringe (BD Plastipak, Madrid, Spain) with a 27-gauge, 7/8 in. needle (Rose GmbH, Trier, Germany) via the hindmost proleg. Bacterial suspensions were supplemented with antibiotics when appropriate to prevent contamination. The injection area was disinfected with a cotton swab soaked in ethanol. Ten healthy, randomly chosen larvae were injected per strain and the infected larvae were stored in petri dishes at 30°C in the dark. To monitor killing of animals due to physical injury or infection, larvae were injected with 10 μl $MgSO_4$ containing appropriate antibiotics. The number of dead larvae was scored 24 h postinfection. Dead larvae turned black as a result of melanization and did not respond to touch. Experiments with more than one dead larva in the control group were not considered and repeated. Data are mean values of at least three independent experiments.

*C. elegans* killing assays were performed essentially as described by Köthe et al. (26). Briefly, 100-μl overnight cultures of *P. aeruginosa* test strains were plated on nematode growth medium (NGM II) agar plates. After 24 h of incubation, approximately 20 to 40 hypochlorite-synchronized stage L4 larvae were transferred onto each test plate. The number of worms at the time of transfer was determined with a Stemi SV6 microscope (Zeiss, Oberkochen, Germany) at a magnification of 50×. Plates were then incubated at 20°C and scored for live worms at 72 h. *E. coli* OP50 was used as a negative control. Data are mean values of at least three independent experiments. Data were analyzed using ANOVA and planned comparisons.

**Assays of motility and lipolytic, proteolytic, and antifungal activities.** The different phenotypes were tested on the wild-type *P. aeruginosa* PUPa3 strain and its various QS mutants. Swimming assays were performed on 0.3% LB agar plates while swarming assays were performed using M8 medium plates (M9 salts without $NH_4Cl$ [25] supplemented with 0.2% glucose and 0.05% glutamate and containing 0.5% agar) (39). The inoculation was performed with a sterile toothpick dipped in a bacterial suspension with an $OD_{600}$ of 2.7. The swimming zone was measured after 24 h of incubation at 30°C, while swarming plates were incubated at 30°C overnight and then at room temperature for an additional 48 h.

Proteolytic and lipolytic activities were determined in the appropriate indicator plates as previously reported (22).

Screening for antifungal activity was performed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) medium. Pathogens used were *Fusarium graminearum*, *Fusarium verticillioides*, *Bipolaris oryzae*, and *Pyricularia grisea* (obtained from F. Favaron, Department of Agriculture, University of Padova, Padua, Italy). A water suspension of the fungal conidia was inoculated in the

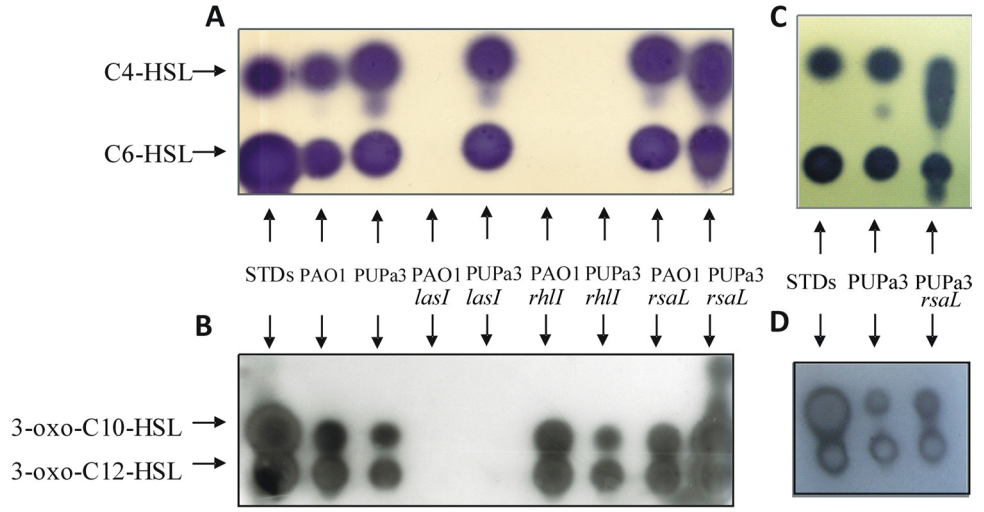


FIG. 1. TLC analysis of AHLs produced by wild type and QS mutants. (A and B) Ethyl acetate extracts of *P. aeruginosa* strain PUPa3 wild type, PAO1 wild type, *lasI* mutants, *rhlI* mutants, and *rsaL* mutants of both PUPa3 and PAO1 strains. Standards (STDs) were synthetic C6-HSL (0.15 nmol) and C4-HSL (0.2 nmol) (for panels A and C) or 3-oxo-C12-HSL (4 nmol) and 3-oxo-C10-HSL (4 nmol) (for panels B and D). (C and D) Ethyl acetate extracts of *P. aeruginosa* strain PUPa3 WT and the *rsaL* mutant. For panels A and C TLCs were overlaid with the bacterial biosensor *C. violaceum* CV026; for panels B and D, TLCs were overlaid with the bacterial biosensor *E. coli*(pSB1075). In all the samples the equivalent of an extraction of $10^9$ cells was run on the TLC, while for the *rsaL* mutant (only in panel D), the equivalent of $10^7$ cells was run, since the *rsaL* mutant produces approximately 100 times more 3-oxo-C12-HSL and 3-oxo-C10-HSL.

melted PDA. The bacterial strains were grown to an $OD_{600}$ of 1.5, and then 0.3 μl was spotted on the PDA plate containing the fungi. The plates were then grown at 25°C for approximately 1 week, during which the fungal mycelia completely covered the agar surface unless mycelial growth was inhibited by the bacteria, in which case we observed an inhibition halo surrounding the bacterial spot.

**Nucleotide sequence accession numbers.** All DNA sequencing was performed either at the CRIBI center (University of Padova, Italy) or at Macrogen, and the nucleotide sequences were deposited in GenBank/EMBL/DDBJ. The Las-QS locus of *P. aeruginosa* PUPa3 is given as a 1,907-bp fragment of pIB103 under the accession number AM778435, and the Rhl-QS locus of *P. aeruginosa* PUPa3 is given as a 2,209-bp XhoI-EcoRV fragment of pIB101 under the accession number AM778436.

## RESULTS

**Identification of the *lasI-lasR* and *rhlI-rhlR* QS genes of *P. aeruginosa* PUPa3 and AHL production.** *P. aeruginosa* PUPa3, just like *P. aeruginosa* PAO1, was found to have two different AHL-dependent QS systems, namely, the LasI/R and the RhlI/R systems. *P. aeruginosa* PUPa3 produces 3-oxo-C12-HSL and its shorter acyl chain derivatives in addition to C4-HSL (Fig. 1A and B). As expected the *lasI* mutant of *P. aeruginosa* PUPa3 no longer produced 3-oxo-C12-HSL but retained the capability of producing the RhlI-synthesized C4-HSL. Similarly, the *rhlI* mutant no longer produced C4-HSL but was still able to synthesize 3-oxo-C12-HSL. These results were the first indication that in *P. aeruginosa* PUPa3 the two QS systems do not cross-regulate each other and thus act independently and not hierarchically, as is the case with *P. aeruginosa* PAO1. The *P. aeruginosa* PUPa3 *lasI rhlI* double mutant no longer produced any AHLs (data not shown). Similarly to the *lasI-lasR* system of *P. aeruginosa* PAO1, PUPa3 also contains a *rsaL* homologue in the intergenic region of these two genes, which is involved in negative regulation of the LasI/R system. The *rsaL* gene was found to affect AHL production of the LasI/R system, since the *rsaL* mutant produced approximately 100 times more LasI-synthesized AHLs (i.e., 3-oxo-C12-HSL and shorter-chain derivatives) while the production of AHLs by the Rhl system remained unchanged (Fig. 1C and D). The presence of two additional spots in the *rsaL* mutant in Fig. 1C is linked to the fact that 3-oxo-C6-HSL and 3-oxo-C8-HSL, which are produced by the Las system in high concentrations in the *rsaL* mutant, are also detected by the *C. violaceum* biosensor. In order to compare AHL thin-layer chromatography (TLC) profiles with that of the well-studied *P. aeruginosa* PAO1 strain, we performed a similar analysis using the PAO1 strain and AHL QS mutant derivatives. As depicted in Fig. 1A, unlike what was observed with strain PUPa3, in the *lasI* mutant of PAO1 no RhlI/R AHLs (i.e., C4- and C6-HSLs) were detected, indicating that under the growth conditions that we used, LasI/R is controlling the RhlI/R system (Fig. 1A and B). Another important difference between PAO1 and PUPa3 was the effect of the RsaL repressor on the production of the LasI/R AHLs. The *rsaL* mutant of PUPa3 resulted in considerable production of 3-oxo-C12-HSL and 3-oxo-C10-HSL, much more than what was observed with the *rsaL* mutant of PAO1 (Fig. 1B). This indicated that RsaL in PUPa3 was much more effective at repressing *lasI* gene expression than the RsaL in PAO1.

**Rice rhizosphere colonization by *P. aeruginosa* PUPa3 and QS mutant derivatives.** As PUPa3 was first isolated from the rhizosphere of rice roots, it was of interest to determine whether the ability to colonize and persist in the rhizosphere of roots was altered in the QS mutants compared to the parent strain. Therefore, we thoroughly and exhaustively investigated root colonization in four independent experiments with rice as host plant. From our first and second root colonization experiments it was evident that the *lasI* mutant, the *lasR* mutant, and the *lasI rhlI* double mutant (DMI) of *P. aeruginosa* PUPa3 were impaired in their root colonization abilities in comparison

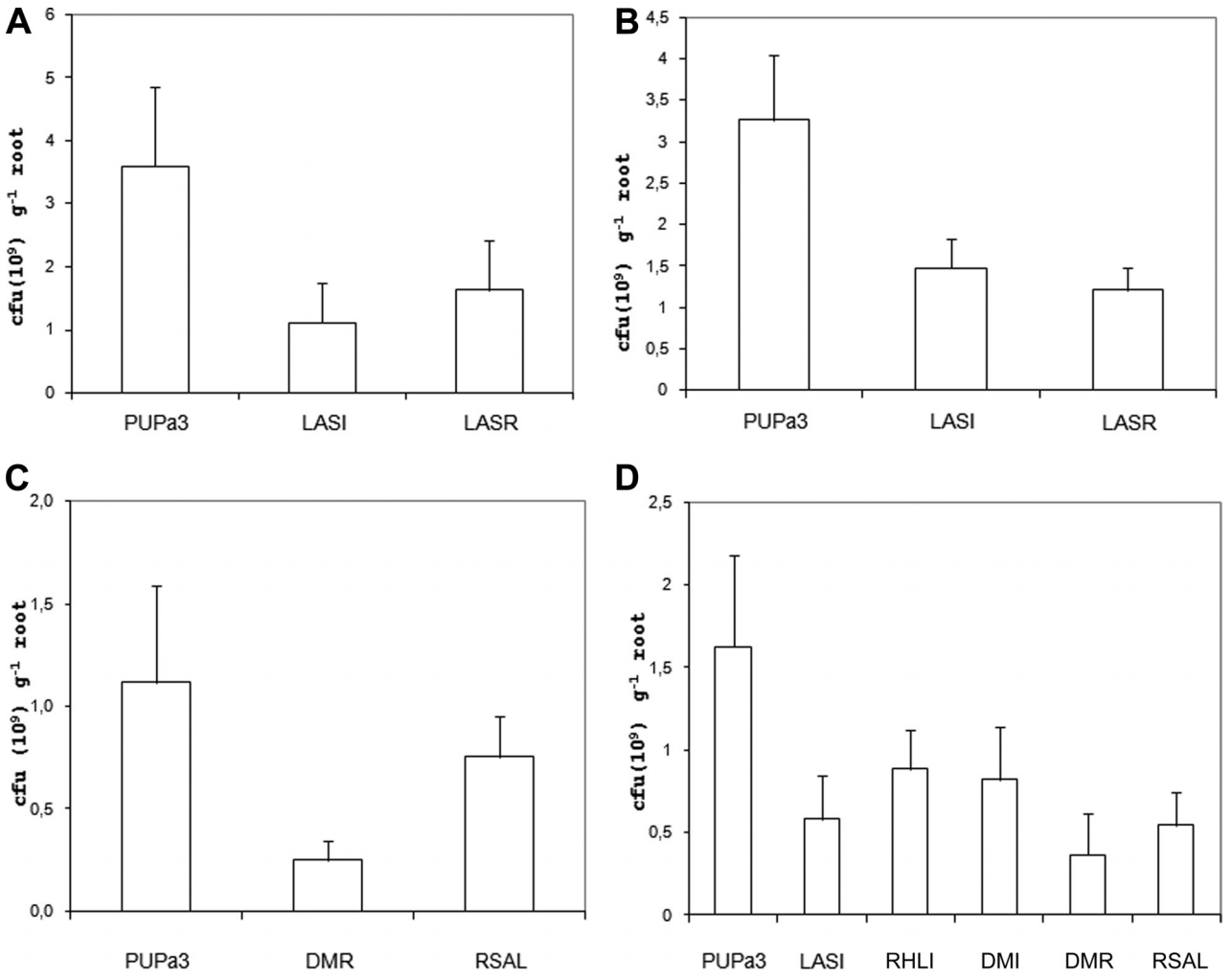


FIG. 2. Root colonization assays of rice rhizosphere *P. aeruginosa* strain PUPa3 and QS mutant derivatives in four independent experiments (A to D). PUPa3, *P. aeruginosa* PUPa3 wild-type strain; LASI, *lasI* mutant; LASR, *lasR* mutant; RHLI, *rhlI* mutant; DMI, *lasI rhlI* double mutant; DMR, *lasR rhlR* double mutant; RSAL, *rsaL* mutant. The colonization of the wild-type strain was significantly higher than that of all QS mutant strains.

to their parent strain (Fig. 2A and B). These observations were supported by two separate statistical analyses performed on each experiment. The first ANOVA, including as independent variables the wild-type PUPa3, the *lasI* mutant LASI, and the *lasR* mutant LASR, yielded a significant main effect [$F(2,21) = 15.56$; $P < 0.0001$]. The root colonization ability was significantly higher in the wild-type strain relative to the two QS mutants [$F(1,21) = 29.88$; $P < 0.0001$]. The second ANOVA, including as independent variables the wild-type PUPa3, and the mutants LASI (*lasI* mutant) and DMI (*lasI rhlI* double mutant), yielded a significant main effect [$F(2,21) = 36.25$; $P < 0.00001$]. The root colonization ability was significantly higher in the wild-type strain relative to both the *lasI* mutant and DMI (*lasI rhlI* double mutant) [$F(1,21) = 71.45$; $P < 0.00001$]. A third experiment compared wild-type PUPa3 root colonization with that of the *lasR rhlR* double mutant (DMR) and RSAL (*rsaL* mutant). An ANOVA with wild-type PUPa3, DMR (*lasR rhlR* double mutant), and RSAL (*rsaL* mutant) as independent variables yielded a significant main effect [$F(2,21) = 16.32$; $P < 0.0001$]. The wild type colonized the root to a significantly higher extent [$F(1,21) = 21.70$; $P < 0.0001$] than the two mutants (Fig. 2C). The fourth experiment was done to further validate the results of the previous experiments and compared root colonization of the wild-type PUPa3 and mutants LASI (*lasI* mutant), RHLI (*rhlI* mutant), DMI (*lasI rhlI* double mutant), DMR (*lasR rhlR* double mutant), and RSAL (*rsaL* mutant). These experiments confirmed that the QS mutant strains all colonize rice roots to a lesser extent than the wild-type *P. aeruginosa* PUPa3 (Fig. 2D). The ANOVA yielded a significant main effect [$F(5,54) = 18.47$; $P < 0.0001$]. The colonization of the wild type was significantly higher than that of all mutant strains [$F(1,54) = 75.72$; $P < 0.00001$].

**Pathogenicity of *P. aeruginosa* PUPa3 and QS mutant derivatives in *C. elegans* and *G. melonella* infection models.** *P. aeruginosa* can infect several animals, including the nematode *C. elegans* (34) and larvae of the greater wax moth *G. mellonella* (37). It was of interest to determine whether this *P. aeruginosa* rhizosphere isolate is virulent for these two animals

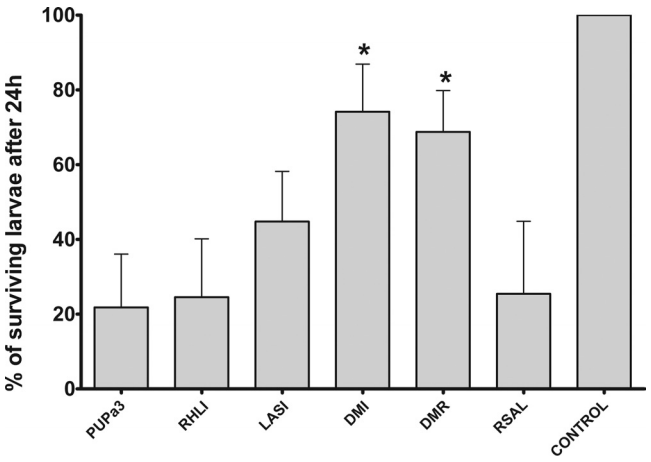


FIG. 3. Killing of *G. mellonella* larvae by *P. aeruginosa* PUPa3, the *rhlI* mutant RHLI, the *lasI* mutant LASI, the DMI (*lasI rhlI*) and DMR (*lasR rhlR*) double mutants, and the *rsaL* mutant RSAL. Larvae were infected with approximately 30 cells of the various *P. aeruginosa* strains and incubated in the dark at 30°C. Dead larvae were determined after 24 h. Data represent means and standard errors of three independent trials. The treatments that showed significant differences (using ANOVA; see text for details) from the wild-type *P. aeruginosa* PUPa3 strain were the two double mutants DMI and DMR and are indicated in the figure by an asterisk.

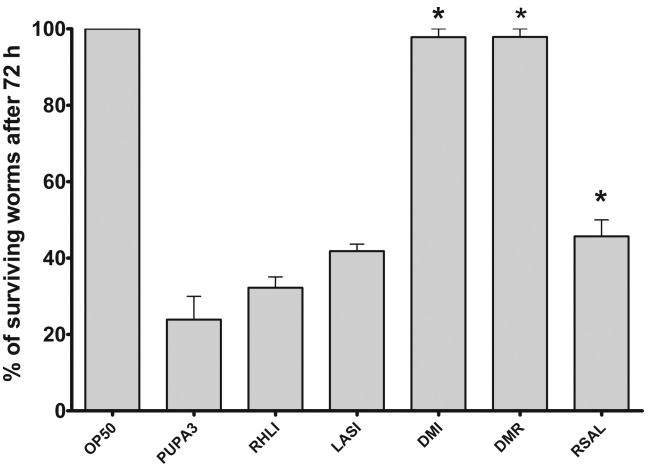


FIG. 4. Killing of *C. elegans* by *P. aeruginosa* PUPa3, the *rhlI* mutant RHLI, the *lasI* mutant LASI, the DMI and DMR double mutants, and the *rsaL* mutant RSAL. *P. aeruginosa* strains were grown on NGM overnight, and 20 to 40 nematodes were then placed onto the plates. Surviving worms were counted after 72 h of incubation at 20°C. Data represent means and standard errors of three independent trials. The treatments that showed significant differences (using ANOVA; see text for details) from the wild-type *P. aeruginosa* PUPa3 strain were the two double mutants DMI and DMR and the *rsaL* mutant and are indicated in the figure by an asterisk.

and if so whether QS plays a role in pathogenesis, as has been previously demonstrated for clinical isolates (for a review, see reference 33). *P. aeruginosa* PUPa3 was found to be very pathogenic for *G. mellonella*, with approximately 30 bacteria being sufficient to kill most larvae within 24 h. The ANOVA yielded a significant main effect [$F(5,15) = 4.90$; $P < 0.01$]. Virulence of the *rhlI* mutant RHLI was virtually indistinguishable from that of the wild type [$F(1,3) = 0.35$; $P = 0.59$], and the *lasI* mutant LASI virulence was found to be only slightly reduced, with a difference that was not significant [$F(1,3) = 4.51$; $P = 0.12$]. However, inactivation of both QS systems, as was the case with the two double mutants, greatly reduced pathogenicity [$F(1,3) = 38.80$; $P < 0.01$] (Fig. 3). When *P. aeruginosa* PUPa3 was used as a food source for *C. elegans* on NGM approximately 80% of the nematodes died within 72 h. The ANOVA yielded a significant main effect [$F(5,10) = 37.72$; $P < 0.0001$]. Inactivation of *rhlI* did not significantly reduce pathogenicity [$F(1,2) = 1.22$; $P > 0.38$], and inactivation of *lasI* only slightly reduced pathogenicity [just missed the significance level; $F(1,2) = 17.38$; $P = 0.052$], whereas both the DMI (*lasI rhlI*) and DMR (*lasR rhlR*) were avirulent [$F(1,2) = 80.01$; $P < 0.05$] (Fig. 4). These results show that for full virulence in the two infection models both QS systems have to be intact, and thus the results further demonstrate that the two systems are not hierarchically arranged.

Interestingly, the RsaL repressor of the LasI/R system did not play a role in virulence in the wax moth model [$F(1,3) = 0.02$; $P = 0.88$], whereas the *P. aeruginosa rsaL* mutant was slightly attenuated in pathogenicity against *C. elegans* [$F(1,2) = 72.84$; $P < 0.05$].

**Role of QS regulation of motility and lipase, protease, and antifungal activities.** It was of interest to determine whether other important colonization-related phenotypes were regulated by QS in *P. aeruginosa* PUPa3. Motility assays were performed, and these showed that swimming motility of all the mutants tested (LASI, RHLI, DMI, DMR, and RSAL) was impaired relative to the wild type, implying that both the Las and the Rhl QS systems control this type of motility (Table 2). The wild-type phenotype was restored in the *lasI* mutant, the *rhlI* mutant, and *lasI rhlI* double mutant by addition of 3-oxo-C12-HSL, C4-HSL, and both these AHL molecules, respectively, to the medium. Swarming was found to be also positively QS regulated in *P. aeruginosa* PUPa3. The wild-type strain formed a typical star shape with several tendrils, the LASI (*lasI*) mutant swarmed but to a lesser extent than the wild type, the RHLI (*rhlI*) mutant swarmed even less than the LASI (*lasI*), and the double mutant DMI (*lasI rhlI*) did not swarm at all (data not shown). It was therefore evident that swarming in

TABLE 2. Phenotypes controlled by QS in *P. aeruginosa* wild type (PUPa3), mutants LASI, RHLI, DMI, DMR, and RSAL, and mutants complemented with AHLs[a]

| Strain | Activity[b] for strain | | |
|---|---|---|---|
| | SW (cm) | LI (mm) | PR (mm) |
| PUPa3 | 7.67 ± 1.15 | 1.07 ± 0.15 | 3.33 ± 0.58 |
| LASI | 3.27 ± 0.90 | 0.50 ± 0.00 | 0.67 ± 0.29 |
| LASI + OC12 | 7.83 ± 1.26 | 0.97 ± 0.15 | 3.17 ± 0.29 |
| RHLI | 3.70 ± 1.85 | 0.47 ± 0.12 | 3.67 ± 0.58 |
| RHLI + C4 | 7.33 ± 2.89 | 0.80 ± 0.20 | 4.00 ± 1.00 |
| DMI | 2.37 ± 1.03 | 0 | 0.33 ± 0.58 |
| DMI + OC12 + C4 | 6.77 ± 3.04 | 0.73 ± 0.06 | 3.00 ± 0.00 |
| DMR | 2.43 ± 0.25 | 0 | 0 |
| RSAL | 3.07 ± 1.36 | 0.8 ± 0.10 | 3.00 ± 0.00 |

[a] Abbreviations for AHLs: C4, C4-HSL; OC12, 3-oxo-C12-HSL.
[b] SW, swimming activity; LI, lipase activity; PR, protease activity. Values are means ± standard deviations.

TABLE 3. Antifungal activities of *P. aeruginosa* PUPa3 wild type and QS mutant derivatives on *B. oryzae*, *F. graminearum*, *F. verticillioides*, and *P. grisea*

| *P. aeruginosa* strain | Antifungal activity against[a]: | | | |
|---|---|---|---|---|
| | *B. oryzae* | *F. graminearum* | *F. verticillioides* | *P. grisea* |
| PUPA3 | +++ | ++ | ++ | +++ |
| LASI | + | + | + | +++ |
| RHLI | + | ++ | +/− | ++ |
| DMI | − | − | − | + |
| DMR | − | − | +/− | − |
| RSAL | − | + | + | + |

[a] −, no inhibition halo; +/−, 2-mm halo; +, 4-mm halo; ++, 6-mm halo; +++, 8-mm halo.

*P. aeruginosa* PUPa3 requires both the LasI/R and RhlI/R systems.

Secreted proteolytic and lipolytic activities were tested, and it was observed that *P. aeruginosa* PUPa3 displayed strong proteolytic activity which was reduced approximately sevenfold in both the *lasI* mutant and the *lasI rhlI* double mutant, while in the *rhlI* mutant the activity did not differ from that of the wild-type parent strain (Table 2). Hence, proteolytic activity appears to be only regulated by the LasI/R system and not by the RhlI/R system in *P. aeruginosa* PUPa3. Lipolytic activity, on the other hand, was reduced to approximately one-half in both the *lasI* and the *rhlI* mutants, and this activity was completely abolished in the *lasI rhlI* double mutant (Table 2). It was therefore concluded that lipolytic activity in *P. aeruginosa* PUPa3 is regulated cooperatively by the two QS systems.

Antifungal activities were tested in wild-type *P. aeruginosa* PUPa3 and its QS mutant derivatives against four different fungi: *Bipolaris oryzae*, *Fusarium graminearum*, *F. verticillioides*, and *Pyricularia grisea*. The strongest antifungal activities were observed against *B. oryzae* and *P. grisea*, and in all cases the activities were found to be regulated by QS (Table 3). Figure 5 summarizes our results on QS-regulated phenotypes in *P. aeruginosa* PUPa3.

## DISCUSSION

The most-studied *P. aeruginosa* strains are clinical isolates, mostly isolated from patients with cystic fibrosis or other immunodeficiency diseases. Considerably less information on the organism's genetics, molecular biology, and lifestyle is available from the environmental strains of *P. aeruginosa*. Pirnay et al. (41) studied the presence and types of *P. aeruginosa* in a river and found that the river community was almost as diverse as the global *P. aeruginosa* population; these data imply that rivers may be sources of distribution of potentially pathogenic *P. aeruginosa* strains. A recent study demonstrated the presence of *P. aeruginosa* strains also in the ocean and concluded from multilocus sequence typing analysis that these strains have diverged from other terrestrial isolates and form a distinct cluster. However, a different study that utilized multilocus sequence analysis on a large number of *P. aeruginosa* strains showed that the environmental isolates clustered with clinical isolates (9). This close relationship between clinical and environmental strains implies that the environment can be a reservoir for opportunistic human pathogens, especially the rhizosphere, where the characteristics that make a bacterial strain an efficient plant growth promoter (e.g., antagonistic properties, colonization ability) could also make it a threatening human opportunistic pathogen (4). *P. aeruginosa* has been isolated from roots of different plants, such as oilseed rape, potato, and rice (17, 27, 38, 55). However, few studies have specifically tested the abilities of these strains to act as root colonizers and plant growth promoters (13). De Vleesschauwer et al. (11) studied the mechanisms of induced systemic resistance in rice as a consequence of root colonization by *P. aeruginosa* strain 7NSK2. In contrast to the little information available on the beneficial interactions between environmental strains and plants, most published work thus far has focused on the ability of clinical isolates to cause disease in plants. For example, Walker et al. showed how *P. aeruginosa* PAO1 and PA14 colonized the roots of *Arabidopsis* and sweet basil, forming a biofilm and eventually killing the plants (59).

Our study analyzed for the first time the QS circuitry of an environmental *P. aeruginosa* strain, PUPa3, and in contrast to previous studies which demonstrated the importance of QS only for pathogenicity in various models, we showed also the involvement of QS in the regulation of beneficial traits, particularly for the interaction with the roots of rice plants. The two AHL QS systems of clinical isolate *P. aeruginosa* strain PAO1 (45) are organized in a hierarchical manner such that the LasI/R exerts transcriptional control over the RhlI/R system (29, 56). The Las and Rhl systems have, however, also been shown to be regulated by several environmental conditions, bringing into question the concept of an organized hierarchy between the two systems (12). Importantly, in contrast to

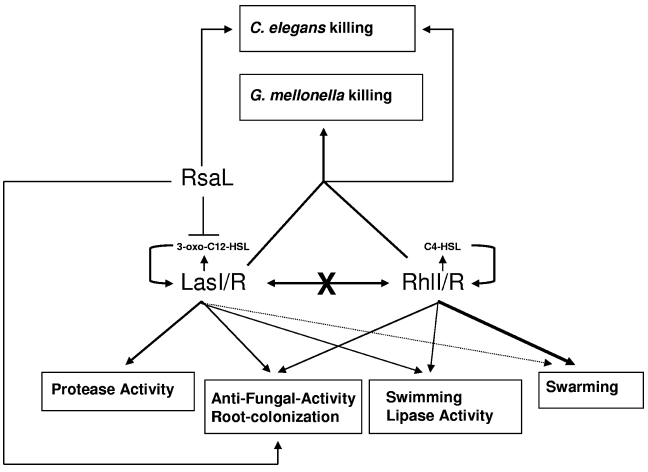


FIG. 5. Working model for the role of QS in *P. aeruginosa* PUPa3. Both QS systems positively regulate swimming, swarming (with the Rhl system being more important than the Las system), lipase, root colonization, and antifungal activity; the LasI/R system positively regulates protease activity, while RhlI/R does not regulate this activity. The two systems acting independently of each other and are not hierarchically organized. The LasI/R system produces 3-oxo-C12-HSL and undergoes positive autoregulation. The RhlI/R system produces C4-HSL and C6-HSL (C4 is the cognate AHL), and it also undergoes positive autoregulation. Both systems together are necessary for the infection of *C. elegans* and *G. mellonella*, whereas both independently are important for rhizosphere colonization. RsaL is a negative regulator of the LasI/R system and it is important for nematocidal killing, for antifungal activity, and for root colonization.

what occurs in PAO1, in *P. aeruginosa* PUPa3 under the growth conditions we used, the LasI/R and RhlI/R QS systems are not hierarchically arranged but act independently and often autonomously regulate the same functions.

As *P. aeruginosa* PUPa3 was isolated from rice roots and had previously been described as a plant growth-promoting rhizobacteria (PGPR), we were interested in determining whether PGPR traits were under QS regulation. We therefore thoroughly tested root colonization of the different strains and showed that this trait is under positive QS regulation in *P. aeruginosa* PUPa3. This is similar to the results found for *P. fluorescens* 2P24, in which the QS mutant also showed a reduced capability for root colonization (60). Another report showing positive QS regulation of root colonization was for PGPR *Pseudomonas chlororaphis* strain 30-84; for this species it was suggested that production of phenazines, which are antibiotics produced by this strain, is under QS regulation. More recently it has been shown that phenazines may also have additional functions, such as being involved in attachment or for themselves serving as additional signals triggering factors important for biofilm formation on the root surface (31). Root colonization can be also negatively controlled by QS, as is the case for the rice rhizosphere *Pseudomonas putida* strain RD8MR3 (51).

In this study the *rsaL* repressor mutant of *P. aeruginosa* was tested for the first time in in vivo models. While *rsaL* is a negative regulator for the production of AHL signaling molecules, the effect of a mutation in this gene was similar to that of mutants in the AHL synthase genes, i.e., all mutants were impaired in their ability to colonize the roots. Therefore, it appears not only that signaling molecules have to be present for efficient root colonization but also that they have to be present in the correct concentration, and *rsaL* provides homeostasis by limiting AHL production to a physiological level, as had been shown previously in in vitro studies (42). RsaL may also interact directly with the promoters of genes linked to root colonization, as it affects gene expression also by acting as a transcriptional regulator independently of AHL production (42).

An important trait for root colonization in rhizosphere bacteria is motility, which allows the colonization of a larger area on the root surface. It was reported that enhanced motility of *P. fluorescens* F113 is an advantageous trait since hypermotile variants were selected during rhizosphere colonization (35). QS mutants of *P. aeruginosa* PUPa3 were partially impaired in swimming motility, hence the QS regulation of swimming motility was found to correlate with that of root colonization, possibly indicating that this type of movement may be important for efficient root colonization. Swarming is a different type of bacterial translocation, by which bacteria can spread as a biofilm over a surface. Similar to PAO1, swarming motility of *P. aeruginosa* PUPa3 was also found to be positively regulated by both QS systems. Another important characteristic of biocontrol bacteria is the ability to protect crops from fungal diseases through the production of antifungal compounds. *P. aeruginosa* PUPa3 possesses antifungal activity and QS was shown to be involved in its regulation. It was, however, observed that the QS systems involved in this regulation depended on the fungal species to which strain PUPa3 was exposed. This result suggests that the two QS systems may independently regulate the production of various factors having a different effect on diverse fungal species. Secreted proteases and lipases also contribute to the antifungal activity of bacterial rhizosphere biocontrol strains. *P. aeruginosa* PUPa3 exhibited lipolytic activity which was dependent on both QS systems. Proteolytic activity of this strain, on the other hand, was found to be regulated only by the LasI/R system. Other reports have demonstrated that lipases and proteases are also regulated by QS in *Pseudomonas* and *Burkholderia* species. However, it is not yet known whether this occurs directly or indirectly (2, 10, 62).

Kumar et al. (28) proposed *P. aeruginosa* PUPa3 as an isolate for potential use as a biofertilizer and antagonist against phytopathogenic fungi, as it had PGPR traits and was isolated from the rhizosphere. In this study we tested whether a *P. aeruginosa* PUPa3 isolate beneficial to plants was pathogenic in other models and therefore could be a threat as an opportunistic human pathogen. Importantly, it was established that *P. aeruginosa* PUPa3 was pathogenic in both the *C. elegans* and the *G. mellonella* wax moth model, indicating that it may behave as an opportunistic human pathogen. In addition, we established that QS was important for virulence in these two models. In both models significant attenuation in pathogenicity was only observed when both QS systems were inactivated, implying that both are independently involved in the regulation of virulence factors. This is interesting, as it could indicate that the virulence factors are independently regulated by the two systems and that only with both QS systems being nonfunctional would their levels be low enough to result in a significant decrease in pathogenicity. This is what occurs with lipase activity, since it is reduced to approximately one-half when either the Rhl or Las systems are inactivated and disappears when both systems are knocked out. The RsaL repressor mutant on the other hand did not affect pathogenicity of strain PUPa3 in the *G. mellonella* model, whereas it was somewhat less infectious in *C. elegans*. RsaL is therefore involved in the regulation of virulence factors for *C. elegans* infection; importantly, it has been shown that RsaL can regulate expression of certain genes independently of QS (42). In summary, the QS systems of the beneficial rhizosphere *P. aeruginosa* strain PUPa3 were shown to act independently, and they are important for rhizosphere colonization and act in concert for animal virulence (Fig. 5 summarizes the role of LasI/R and RhlI/R in strain PUPa3). This study also highlights that the role and mode of action of QS systems in *P. aeruginosa* may vary in different strains, since several aspects of QS observed in strain PUPa3 are different from what is known for *P. aeruginosa* PAO1.

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# Appendix 4

## *Burkholderia cenocepacia* J2315 acyl carrier protein: A potential target for antimicrobials' development?

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# *Burkholderia cenocepacia* J2315 acyl carrier protein: A potential target for antimicrobials' development?

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Biofilm
*Caenorhabditis elegans* model of infection

ABSTRACT

This work describes the isolation and characterization of an acyl carrier protein (ACP) mutant from *Burkholderia cenocepacia* J2315, a strain of the *Burkholderia cepacia* complex (Bcc). Bcc comprises at least 9 species that emerged as opportunistic pathogens able to cause life-threatening infections, particularly severe among cystic fibrosis patients. Bacterial ACPs are the donors of the acyl moiety involved in the biosynthesis of fatty acids, which play a central role in metabolism. The mutant was found to exhibit an increased ability to form biofilms in vitro, a more hydrophobic cell surface and reduced ability to colonize and kill the nematode *Caenorhabditis elegans*, used as a model of infection. The *B. cenocepacia* J2315 ACP protein is composed of 79 amino acid residues, with a predicted molecular mass and p*I* of 8.71 kDa and 4.08, respectively. The ACP amino acid sequence was found to be 100% conserved within the genomes of the 52 *Burkholderia* strains sequenced so far. These data, together with results showing that the predicted structure of *B. cenocepacia* J2315 ACP is remarkably similar to the *Escherichia coli* AcpP, highlight its potential as a target to develop antibacterial agents to combat infections caused not only by Bcc species, but also by other *Burkholderia* species, especially *B. pseudomallei* and *B. mallei*.



## 1. Introduction

The *Burkholderia cepacia* complex (Bcc) comprises at least 9 distinct species, which emerged as important opportunistic pathogens, particularly in patients with cystic fibrosis (CF) or chronic granulomatous disease [1,2]. *Burkholderia cenocepacia* and *Burkholderia multivorans* are the predominant species causing human pulmonary infections, although isolates from all the other Bcc species have been also associated with poor clinical outcome [2,3], which is widely variable, ranging from asymptomatic carriage to a rapid and fatal pneumonia (the so-called cepacia syndrome) [1,2]. Most of the cases of patient-to-patient transmission have been caused by *B. cenocepacia* [2,3], in particular by strains of the ET-12 lineage.

A major problem associated with Bcc infections is their intrinsic resistance to a large range of antimicrobials, rendering their eradication very difficult [4]. In a recent systematic comparison of the in vitro antimicrobial susceptibility of Bcc isolates from Portuguese CF patients, more than one-half of the Bcc isolates were multi-drug resistant (MDR) [5]. Furthermore, MDR rates were unevenly distributed among the species studied, with *B. cenocepacia* isolates exhibiting the highest MDR rate [5], in agreement with other studies [1,4,6]. Therefore, new strategies for battling these opportunistic pathogens are urgently needed.

Aiming at the identification of novel antimicrobial resistance and virulence determinants, we have used a plasposon random mutagenesis strategy to construct a mutant library from the ET-12 lineage CF isolate *B. cenocepacia* J2315. Here we describe the isolation and characterization of an acyl carrier protein (ACP) mutant strain from *B. cenocepacia* J2315. Bacterial ACPs are the donors of the acyl moiety involved in the biosynthesis of fatty acids, phospholipids, endotoxins, glycolipids and signalling molecules necessary for growth and pathogenesis [7,8]. The significant differences in organization, structure of enzymes, and role played by fatty acid biosynthesis between bacteria and humans make this system an attractive target for the development of novel antimicrobial compounds [8]. For example, ACP is the cellular target of the pantothenamide class of pantothenate antimetabolites with antibacterial action [9]. The results presented in this study implicate a role for ACP on the pathogenesis of *B. cenocepacia* J2315. The finding that the protein sequence is 100%

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conserved among the 52 *Burkholderia* strains sequenced so far highlights ACP as a good candidate to develop new antimicrobials to combat infections caused not only by members of the Bcc, but also by other *Burkholderia* species, especially by *B. mallei* and *B. pseudomallei*.

## 2. Results and discussion

### 2.1. Identification of a B. cenocepacia J2315 acp mutant

The *B. cenocepacia* SJ1 mutant was identified during the qualitative screening for mutants exhibiting reduced virulence to the nematode *Caenorhabditis elegans*, using the 48-well plate mortality assay [10]. Mutants were derived from *B. cenocepacia* J2315 by random plasposon mutagenesis with pTnMod-OCm [11], based on the methods previously described [12]. The presence of a single plasposon insertion within the genome of mutant SJ1 was confirmed by Southern blot, using as probe the 1.63 kb DNA fragment containing the chloramphenicol resistance cassette obtained by restriction of pTnMod-OCm with *Xba*I (data not shown). Total DNA from the mutant was isolated, digested with *Bam*HI, self-ligated and used to transform *Escherichia coli* DH5α, using standard procedures [13]. The DNA insert from the plasmid recovered was PCR sequenced using primers CmF (5′-GCG GCC GCA CTT GTG TAT AA-3′) and CmI (5′-TAC CGT CGA CAT GCA TGG CG-3′). Results revealed that the plasposon interrupted an ORF putatively encoding an acyl carrier protein (ACP), designated *acp*, containing the carrier protein superfamily signature motif **$^{33}$LGADSL$^{38}$**, with the serine residue required for phosphopantetheinylation at position 37 (Fig. 1A). ACPs have a crucial role on fatty acid biosynthesis and are functional only after post-translational covalent attachment of a 4′-phosphopantetheinyl moiety from CoA to the serine residue [8]. In Gram-negative bacteria, ACPs and acyl-ACP intermediates also participate in the synthesis of the β-hydroxy fatty acids of lipid A and of the acylated homoserine lactones (AHLs) involved in quorum-sensing [7,8]. The *B. cenocepacia* J2315 ACP amino acid sequence was found to be conserved in several Gram-negative bacteria (Fig. 1A), and, in particular, it is 73% identical to the *E. coli* K12 *acpP* gene product. Similar to the *E. coli* AcpP, which has 8847 Da and a p*I* of 4.1 [8], the *B. cenocepacia* J2315 ACP has a predicted molecular mass and p*I* of 8.71 kDa and 4.08, respectively.

### 2.2. The acp gene locates in a fab cluster, duplicated in the B. cenocepacia J2315 chromosome 1

In *E. coli*, the *acpP* gene is essential, and only recently conditionally defective *acpP* mutants were reported [14]. In contrast, no growth defects could be observed during batch growth of *B. cenocepacia* mutant SJ1 (Fig. 2A). Therefore, a search within the genome sequence of *B. cenocepacia* J2315 for genes homologous to *acp* was performed. This analysis revealed the presence of a second putative *acp*-encoding gene within the genome sequence of *B. cenocepacia* J2315, which is 100% identical to the *acp* sequence identified initially. Both genes are located on chromosome 1, and span nucleotide positions 1083131–1083370 (gene BCAL0995) and 3155881–3155642 (gene BCAL2875), respectively (Fig. 1B). Both *acp*-encoding genes were found to be part of a duplicated gene cluster, containing the *E. coli fab* (fatty acid biosynthesis) cluster homologues *plsX*, *fabH*, *fabD*, *fabG*, *acp*, and *fabF* (Fig. 1B). In *E. coli*, genes *fabD*, *fabF*, *fabG*, and *fabH* encode, respectively, malonyl-CoA-ACP transacylase, β-ketoacyl-ACP synthase II, β-ketoacyl-ACP reductase, and β-ketoacyl-ACP synthase III [8]. Although *plsX* remains poorly characterized, it was recently suggested that PlsX plays a role in 1-acyl-glycerol-3-phosphate metabolism and, possibly, on the regulation of the intracellular concentration of acyl-ACP [15]. Further inspection of the *B. cenocepacia* J2315 chromosome 1 also revealed that the repeated region containing the *fab* cluster is 57,050 nucleotides (nt) long, spanning nt 1056864–1113819 (region 1) and 3125194–3182148 (region 2) (Fig. 1B). Inspection of the available genome sequences of other *Burkholderia* strains revealed that this duplication is unique in the *B. cenocepacia* J2315 genome.

### 2.3. Phenotypic characterization of the B. cenocepacia J2315 acp mutant

The fatty acid composition of *B. cenocepacia* J2315 and its derivative mutant SJ1 was compared (Table 1). Results obtained indicate that, compared to the wild-type strain, the relative amount of saturated and unsaturated C-16 fatty acids is about 5% increased when compared to C-17 and C-18 saturated and unsaturated fatty acids, suggesting that the biosynthesis of fatty acids of longer chain is slightly reduced in the mutant strain. The ability of the two strains to form biofilms in microtiter plates was also compared, using S medium, previously shown to lead to thicker biofilms [16].

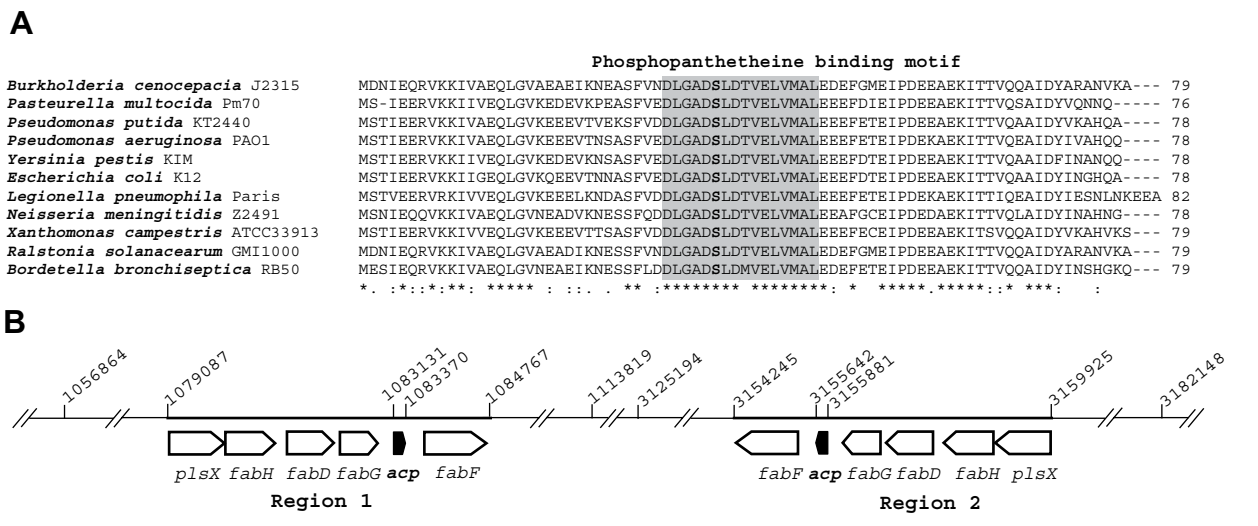


**Fig. 1.** (A) Comparison of the ACP protein of *B. cenocepacia* J2315 with ACPs from the indicated strains. Identical amino acid residues are indicated with asterisks, conserved and semi-conserved substitutions are indicated with 2 dots or a single dot, respectively. The phosphopantetheine binding motif is boxed in grey. Alignments were generated with ClustalW [26]. (B) Physical organization of the two repeated *fab* clusters, located at the indicated nucleotide positions in chromosome 1 of *B. cenocepacia* J2315.

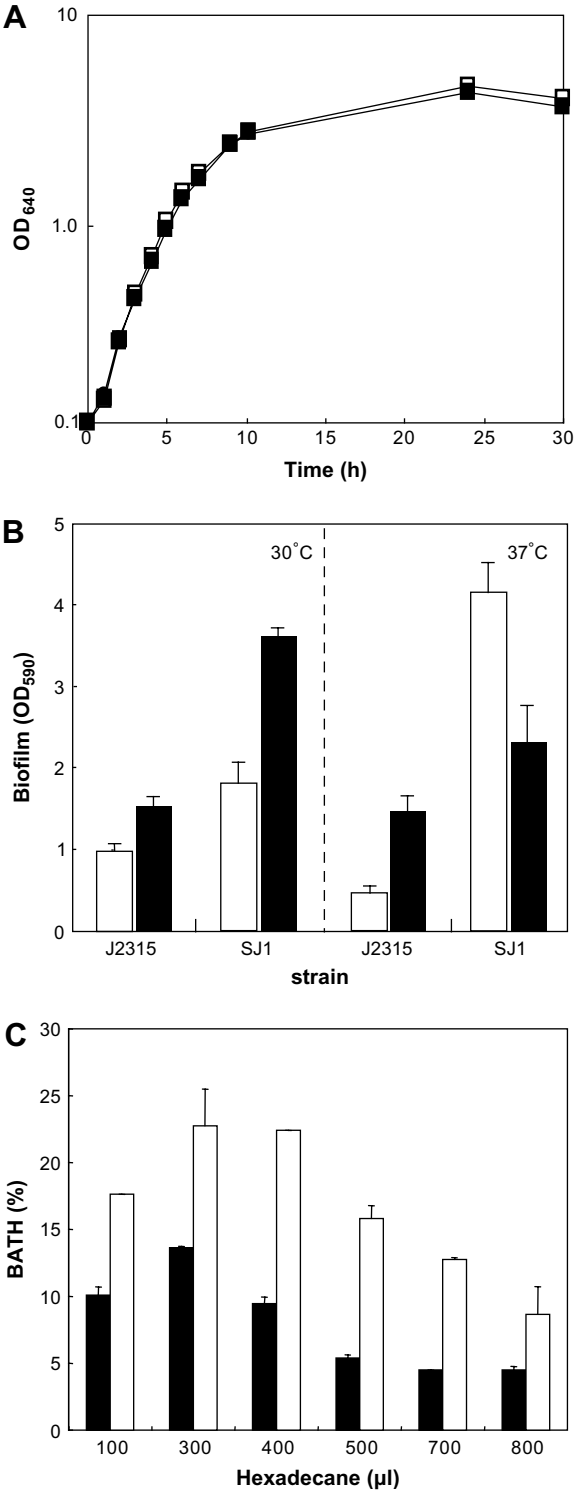


**Fig. 2.** Comparison of the *B. cenocepacia* strains J2315 (■) and mutant SJ1 (□) (A) batch growth curves in LB medium with agitation at 37 °C, (B) biofilm formation ability in S liquid medium, and (C) cell hydrophobicity. In panel B, open bars and closed bars represent the amount of biofilm formed after 24 or 48 h, respectively, by the indicated strains, at 30 or 37 °C.

When compared to the wild-type strain *B. cenocepacia* J2315, the mutant strain formed significantly thicker biofilms in vitro after 24 or 48 h, at the temperatures of 30 or 37 °C (Fig. 2B). A possible explanation for the ability of the mutant strain to form thicker biofilms when compared to the wild-type strain is the increased hydrophobicity of the mutant cells' surface, which adhered at a higher extent to the solvent hexadecane when compared to cells of the wild-type strain (Fig. 2C). It is possible that the increased cell surface hydrophobicity observed in the case of the mutant SJ1 might result from altered outer membrane topology [17].

**Table 1**
Total fatty acid content (as percentage of total fatty acids) of *B. cenocepacia* J2315 and the mutant SJ1.

| Fatty acid (%) | C16:0 | C16:1 (ω 7*c* + ω 6*c*) | C17:0 CYC | C18:1 (ω 7*c*) |
|---|---|---|---|---|
| *B. cenocepacia* J2315 | 23.2 ± 0.2 | 15.1 ± 0.1 | 10.2 ± 0.1 | 28.5 ± 0.2 |
| *B. cenocepacia* SJ1 | 24.2 ± 0.2 | 18.5 ± 0.2 | 9.2 ± 0.1 | 24.9 ± 0.2 |

Abbreviations: c, *cis*; CYC, cyclopropane.

### 2.4. *The* B. cenocepacia *J2315* acp *mutant shows decreased ability to colonize and kill the nematode* C. elegans

The ability of *B. cenocepacia* J2315 and the mutant SJ1 to kill the nematode *C. elegans* was compared using slow-killing experiments. In these experiments, we have used the *C. elegans* mutant strain DH26 which is unable to reproduce at 25 °C, thus facilitating the counting of nematodes without the interference of progeny. Under these experimental conditions, the mutant exhibited a significant reduction of its ability to kill the nematodes (Fig. 3A), pointing out a role for *acp* in the virulence of *B. cenocepacia* J2315. We have also investigated the ability of *B. cenocepacia* J2315 and the *acp* mutant strain to colonize the nematode intestinal tract by determining the number of colony-forming units (CFUs) in suspensions resulting

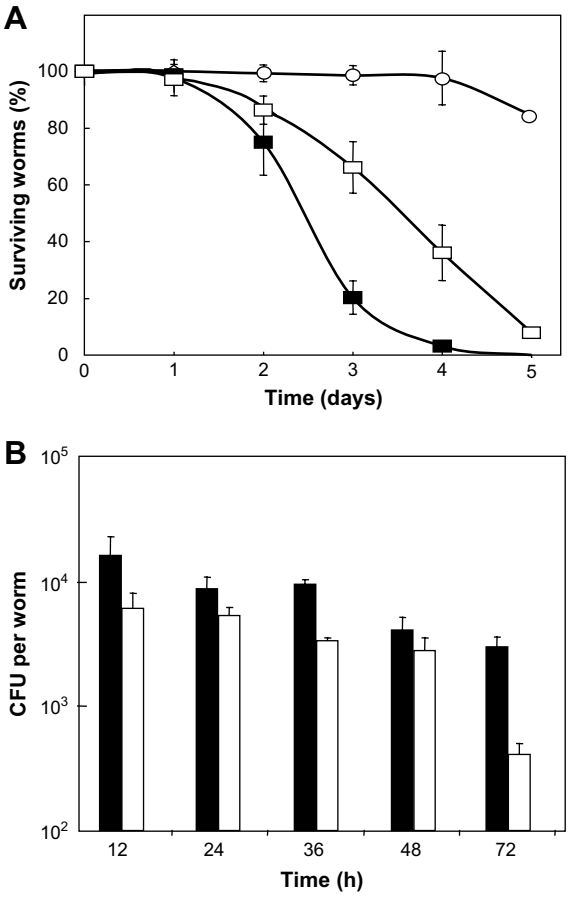


**Fig. 3.** Comparison of the *B. cenocepacia* strains J2315 (■) and mutant SJ1 (□) (A) ability to kill the nematode *C. elegans* and, (B) colony-forming units in the nematode intestinal tract, after infection for the indicated time. In panel A, open circles represent surviving worms feeding in *E. coli* OP50.

from vortexing infected worms with 1.0 mm silicon carbide particles. As shown in Fig. 3B, the total CFUs per worm were significantly higher when worms were fed with the wild-type strain *B. cenocepacia* J2315, especially at 72 h post-infection. The infection of *C. elegans* was also monitored microscopically by using DsRed-tagged variants of the wild-type and mutant strain. In agreement with the CFUs measurements, a slight reduction in the amount of bacterial cells in the nematodes gut was observed with the mutant strain relative to the wild-type (data not shown). However, this experiment also showed that the mutant is not per se defective in gut colonization. Taken together, these results reinforce the observed reduced ability of the mutant to kill the nematode *C. elegans*, pointing out that *acp* plays a role on the virulence of *B. cenocepacia* J2315.

Since ACP is also involved in the formation of AHLs, we have investigated the ability of both strains to produce AHLs using the GFP-based biosensor *Pseudomonas putida* F177(pAS-C8), which is most sensitive for *N*-octanoyl homoserine lactone (C8-HSL) [18], the major AHL produced by *B. cenocepacia* J2315. However, no differences could be detected, suggesting that the inactivation of the *acp* gene did not affect AHL production by *B. cenocepacia* J2315 (data not shown). This result suggests that ACP contributes to *B. cenocepacia* J2315 virulence through other mechanism(s) besides quorum-sensing.

### 2.5. *ACPs from sequenced strains of the* Burkholderia *genus are 100% identical*

The presence of *B. cenocepacia* J2315 ACP homologues within the genomes of other *Burkholderia* strains was investigated. Remarkably, with the exception of the ACP putative protein from *B. mallei* ATCC 10399, with a valine instead of an isoleucine at position 70, all the other 50 genome sequences of *Burkholderia* strains encode putative ACP proteins, which are 100% identical to the *B. cenocepacia* J2315 ACP described in this work. This includes *B. multivorans* ATCC 17616; *B. cenocepacia* strains AU 10546, HI2424, MC0-3, and PC184; *B. ambifaria* strains MC40-6 and AMMD; *B. dolosa* AUO158; *B. vietnamiensis* G4; *B. mallei* strains 2002721280, ATCC 23344, FMH, GB8 horse 4, JHU, NCTC 10229, NCTC 10247, SAVP1, and PRL-20; *B. pseudomallei* strains 1106a, 1106b, 1655, 1710a, 1710b, 305, 406e, 668, K96243, Pasteur, S13, 112, 14, 7894, 9, 91, B7210, BCC215, DM98, and NCTC 13177; *Burkholderia* sp. 383; *B. thailandensis* E264, BT4, MSMB43, ATCC 700388, and TXDOH; *B. phymatum* STM815; *B. phytofirmans* PsJN; *B. oklahomensis* strains EO147 and C6786; *B. ubonensis* Bu; and *B. xenovorans* LB400. These results strongly suggest that this protein is highly conserved within bacteria of the *Burkholderia* genus. This analysis was extended to the other genes of the duplicated *fab* cluster identified in this work. Results obtained revealed that for the putative proteins PlsX, FabH, FabD, FabG, and FabF, the percentages of identity of orthologous proteins within the *Burkholderia* genus ranged from 99 to 86%, 99 to 88%, 99 to 85%, 99 to 89%, and 99 to 81%, respectively (data not shown). These observation highlights ACP as a singularly conserved protein within this bacterial genus.

### 2.6. B. cenocepacia *J2315 ACP protein is structurally similar to the* E. coli *AcpP*

Since the ACP protein identified in this work is common to all the sequenced strains of the *Burkholderia* genus, the 3-D structure of ACP was predicted using the SWISS MODEL Server using as template the crystal structure of AcpP from *E. coli* K12, resolved at 1.55 Å (PDB entry 2FaeB). Remarkably, ACP was predicted to be composed of 4 α-helices, arranged similarly to the *E. coli* protein AcpP (Fig. 4). In the *E. coli* AcpP, the 3 major α-helices (I, II, and IV) form a hydrophobic pocket enclosing the thioesther-linked acyl group attached to the

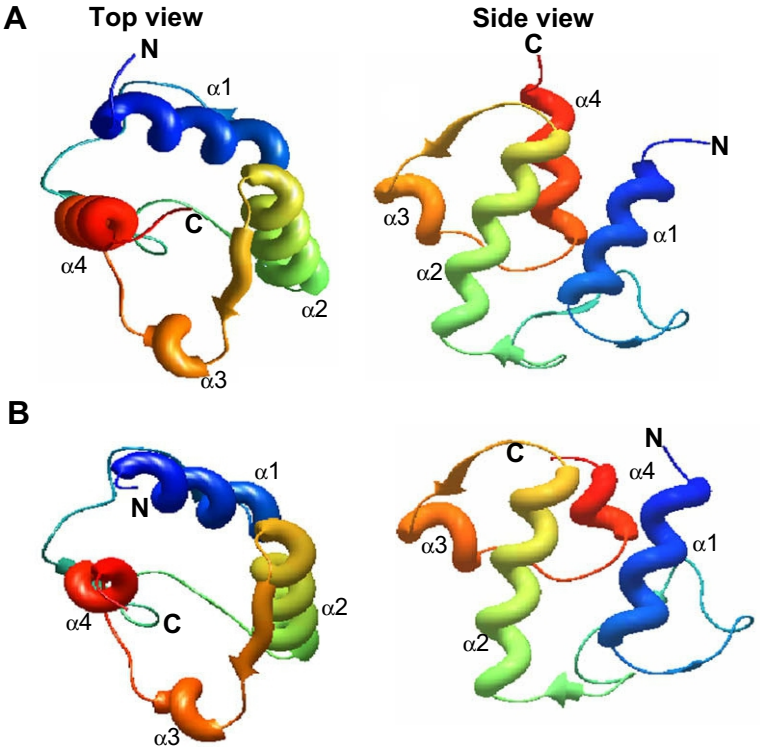


**Fig. 4.** (A) *Escherichia coli* AcpP structure model, based on the X-ray crystal structure (resolution: 1.55 Å) [19]; (B) structural model of the *B. cenocepacia* J2315 ACP protein, modelled using the *E. coli* AcpP as template. Helix α1 of *E. coli* AcpP is formed by residues 3–15 and runs approximately antiparallel to helix α2, which is the longest of the bundle (15 residues, residues 36–50). The short α3 helix (residues 56–61) lies orthogonally to helices α1 and α2. The α4 helix (residues 65–75) runs antiparallel to helix α1, at an angle of approximately 45°. Graphics were generated using the RasWin Molecular Graphics (Windows version 2.7.3.1).

phosphopantetheine prosthetic group. The short α-helix III links helices II and IV [7,19] (Fig. 4). The predicted α-helix IV of the *B. cenocepacia* J2315 ACP is shorter than the corresponding α-helix IV of the *E. coli* AcpP, suggesting that the hydrophobic pocket might accommodate acyl groups with longer chain lengths (Fig. 4).

## 3. Conclusions

In this work we report the identification and phenotypic characterization of an acyl carrier protein mutant from *B. cenocepacia* J2315, encoded by a gene located in a chromosomal segment which is duplicated. Although the mutant exhibited no growth defects, and an increased ability to form thicker biofilms, it showed a reduced ability to colonize and kill *C. elegans*, indicating that ACP is involved in virulence. The fact that the amino acid sequence of ACP is 100% conserved in *Burkholderia* strains sequenced so far, together with the remarkable predicted similarity between the ACP proteins from *B. cenocepacia* J2315 and *E. coli*, highlights its potential as a potential target to develop new antibacterial agents to combat infections caused not only by Bcc species, but also by other *Burkholderia* species, especially by *B. pseudomallei* and *B. mallei*.

## 4. Materials and methods

### 4.1. Bacterial strains, plasmids and culture conditions

The CF isolate *B. cenocepacia* J2315 and its derivative mutant *B. cenocepacia* SJ1 (*acp*::TnModOCm) were used in this work. *E. coli* DH5α was used as host of plasmids. *E. coli* OP50 was used as the nematode *C. elegans* feeding strain. When in use, *B. cenocepacia* strains were maintained in PIA (*Pseudomonas* isolation agar, BD) plates, supplemented with 600 μg/ml chloramphenicol, in the case of mutant *B. cenocepacia* SJ1. *E. coli* strains were maintained in LB (Lennox broth, Sigma) plates, supplemented with 50 μg/ml chloramphenicol when appropriate. Unless otherwise stated, liquid cultures were carried out in LB (Sigma) liquid medium supplemented with the appropriate antibiotics, at 37 °C with orbital agitation (250 rpm). Bacterial growth was followed by measuring culture optical density at 640 nm ($OD_{640}$).

### 4.2. Molecular biology techniques

Total DNA was extracted from exponentially growing liquid cultures of *B. cenocepacia* strains J2315 and SJ1, using the DNeasy Blood &Tissue kit (Qiagen). Plasmid isolation and purification, DNA amplification and restriction, agarose gel electrophoresis, Southern blot experiments, and *E. coli* transformation were carried out using standard procedures [13].

### 4.3. Nucleotide and amino acid sequence analysis and structure prediction

DNA and protein sequences were analysed using bioinformatic tools resident at the National Center for Biotechnology Information (NCBI) or at the ExPASy-Prosite. Searches for homologous sequences within the genomes of *B. cenocepacia* J2315 and other *Burkholderia* strains were performed using the *B. cenocepacia* J2315 genome project database available through http://www.sanger.ac.uk/Projects/B_cenocepacia/, the Integrated Microbial Genomes system [20], and the NCBI Microbial Genomes Draft Assembly (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=2&p1=5:0&p2=2). Three-dimensional structural modelling was performed using the Swiss-model platform [21], using the *E. coli* decanoyl-ACP (PDB entry code 2FaeB) as template. The model data were used to generate the predicted graphical structures using RasWin Molecular Graphics (Windows version 2.7.3.1).

### 4.4. Fatty acid composition

Whole cell fatty acids from cells of *B. cenocepacia* strains J2315 and SJ1, grown on the surface of TSA (tryptic soy agar, Difco) for 24 h at 28 °C, were analysed by gas chromatography (GC) of the fatty acid methyl esters according to the standard procedure of the Sherlock® 4.5 Microbial Identification System (MIDI Inc., Newark, DE, USA). Results are the mean values of at least two independent experiments.

### 4.5. Detection and quantification of AHLs

AHL production was investigated by cross-streaking *B. cenocepacia* strains J2315 and SJ1 against the GFP-based AHL biosensor *P. putida* F177(pAS-C8), based on previously described methods [18,22].

### 4.6. Biofilm formation assay

Biofilms formed in vitro by *B. cenocepacia* strains J2315 or SJ1 were quantified based on previously described methods [16]. Results are median values of at least 5 repeats from 3 independent experiments.

### 4.7. Bacterial adhesion to hexadecane

The cell surface hydrophobicity of *B. cenocepacia* strains J2315 and SJ1 was determined using the Bacterial Adhesion to Hydrocarbon (BATH) method [23]. Briefly, cells from liquid cultures carried out for 24 h at 37 °C with agitation were harvested by centrifugation, washed twice with PBS buffer, and resuspended in a volume of PBS calculated to obtain an $OD_{640}$ of 0.6. 1.5 ml aliquots of these cell suspensions were mixed with volumes of hexadecane ranging from 0 to 800 μl, vortexed for 20 s and the phases were allowed to separate for 30 min. After this time, the $OD_{640}$ of the aqueous phase was measured. Results are median values of at least three independent experiments and were expressed as percentage of hydrophobicity: Hydrophobicity (%) = $(1 - OD_{640}$ aqueous phase/$OD_{640}$ initial cell suspension) × 100.

### 4.8. Nematode killing assays and bacterial colonization

Nematode slow-killing assays were performed based on the methods described by Cardona et al. [10], using the *C. elegans* mutant strain DH26 [24]. Briefly, slow-killing assays were performed as follows: 50 μl of overnight cultures of *B. cenocepacia* strains J2315 or SJ1, grown at 37 °C with agitation in LB liquid medium, was spread onto the surface of 35 mm diameter Petri plates containing 4 ml of nematode growth medium II (NGMII) [10] and incubated for 24 h at 37 °C. Approximately 25 hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were inoculated per plate and incubated at 25 °C. The number of live worms was determined using a Stemi 2000-C stereomicroscope at a magnification of 50×. Nematodes were considered dead when they failed to respond to touch. Slow-killing experiments were carried out at least 5 times with 5 plates per experiment. *E. coli* OP50 was used as a negative control.

Bacterial colonization of the digestive tract of nematodes was performed based on the methods described by Moy et al. [25]. Briefly, groups of approximately 10 worms at the L4 larval stage (prepared as described above) were washed 3 times with 250 μl M9 buffer supplemented with 1 mM sodium azide. After washings, 10 worms were picked and resuspended in 250 μl of M9 buffer, and transferred into 2.2 ml Eppendorf tubes containing approximately 400 mg of sterile 1 mm diameter silicon carbide beads (Biospec Products, USA). The tubes were vortexed for 1 min and the resulting

suspension was diluted and plated on PIA to determine colony-forming units. The supernatants resulting from the washing procedure were diluted and plated on PIA plates to assess the CFUs adherent to the worms' cuticle. M9 buffer contained, in gram per liter, $KH_2PO_4$ 3.0, $Na_2HPO_4$ 6.0, NaCl 5.0, and $MgSO_4$ 0.12. Results are the means of triplicates from at least 3 independent experiments.

Infection of *C. elegans* by the wild-type strain and mutant SJ1 was also investigated microscopically. To this end the wild-type and mutant strain were tagged with the red fluorescent protein DsRed by inserting a $P_{lac}$-*dsred*-T0-T1 cassette randomly into the chromosomes of the two organisms. These strains were used as food source for *C. elegans* and infected worms were inspected after 48 and 72 h by the aid of a Leica TCS SPE laser scanning confocal microscope.

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# Appendix 5

**Cystic fibrosis-niche adaptation of *Pseudomonas aeruginosa* reduces virulence in multiple infection hosts.**

Nicola Ivan Lorè, Cristina Cigana, Ida De Fino, Camilla Riva, Mario Juhas, Stephan Schwager, Leo Eberl, Alessandra Bragonzi.

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# Cystic Fibrosis-Niche Adaptation of *Pseudomonas aeruginosa* Reduces Virulence in Multiple Infection Hosts

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## Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* is able to thrive in diverse ecological niches and to cause serious human infection. *P. aeruginosa* environmental strains are producing various virulence factors that are required for establishing acute infections in several host organisms; however, the *P. aeruginosa* phenotypic variants favour long-term persistence in the cystic fibrosis (CF) airways. Whether *P. aeruginosa* strains, which have adapted to the CF-niche, have lost their competitive fitness in the other environment remains to be investigated. In this paper, three *P. aeruginosa* clonal lineages, including early strains isolated at the onset of infection, and late strains, isolated after several years of chronic lung infection from patients with CF, were analysed in multi-host model systems of acute infection. *P. aeruginosa* early isolates caused lethality in the three non-mammalian hosts, namely *Caenorhabditis elegans*, *Galleria mellonella*, and *Drosophila melanogaster*, while late adapted clonal isolates were attenuated in acute virulence. When two different mouse genetic background strains, namely C57Bl/6NCrl and Balb/cAnNCrl, were used as acute infection models, early *P. aeruginosa* CF isolates were lethal, while late isolates exhibited reduced or abolished acute virulence. Severe histopathological lesions, including high leukocytes recruitment and bacterial load, were detected in the lungs of mice infected with *P. aeruginosa* CF early isolates, while late isolates were progressively cleared. In addition, systemic bacterial spread and invasion of epithelial cells, which were detected for *P. aeruginosa* CF early strains, were not observed with late strains. Our findings indicate that niche-specific selection in *P. aeruginosa* reduced its ability to cause acute infections across a broad range of hosts while maintaining the capacity for chronic infection in the CF host.

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## Introduction

*Pseudomonas aeruginosa* is a common bacterium found in a wide range of environments; it infects nematodes, insects, plants, and ameba in the laboratory and probably encounters a similar range of potential hosts in the wild [1]. In humans, *P. aeruginosa* causes a wide range of infections, including deadly pneumonia when infecting immuno-compromised or cystic fibrosis (CF) patients. The clinical outcome of *P. aeruginosa* infection ranges from acute to chronic infections. Individuals in intensive care units can develop ventilator-associated pneumonia and/or sepsis as a result of *P. aeruginosa* infection. Patients with CF develop life-long chronic lung *P. aeruginosa* infection which leads to death.

Genomes of different *P. aeruginosa* isolates share a remarkable amount of sequence similarity when isolated from the environment or from different clinical origins [2,3]. A considerable conservation of genes including nearly all known virulence factors, such as pyocyanin, a type III secretion system (T3SS), several proteases, lipases and phospholipases and rhamnolipids was observed in *P. aeruginosa* strains isolated from the environment, immuno-compromised patients and CF patients at the onset of infection [3]. Despite the overall genome similarity among diverse *P. aeruginosa* strains, point mutations accumulate in bacterial lineages persisting in CF airways. Mutations commonly acquired by *P. aeruginosa* strains during CF chronic infection are those in the regulators of alginate biosynthesis [4] and virulence genes involved in the LPS modification [5], motility [6], in the quorum-sensing regulation [7,8], in biosynthesis of the T3SS [9] and multidrug-efflux pumps, and in mutator genes [10]. Changes in metabolic functions have also been described [11]. In addition, whole genome sequence analysis of *P. aeruginosa* longitudinal strains from the same CF patient revealed that a surprisingly large number of genes in the genome can be targets for mutation during adaptation to CF airways, although only a few of these genes were found to be affected in many of the late isolates [11]. Recent work demonstrated that the greatest contribution to the extremely high levels of genetic diversity is within an individual patient rather than between patients [12].

Pathogenicity of *P. aeruginosa* isolates from different habitats and clinical origin, including complex phenotypes from CF patients,

can be strikingly different. Previous studies in the *P. aeruginosa* reference strains PA14 and PAO1, and additional strains from various sources, showed that the genes required for pathogenicity in one strain are neither required for nor predictive of virulence in other strains [2]. When comparing *P. aeruginosa* strains derived from the same type of infection, there was no consistent clustering with respect to their phenotype in *C. elegans* [2]. For example, urinary tract infection strains exhibited a wide range of virulent and avirulent phenotypes in acute infection models. In the same vein, both the most and the least virulent strains tested were isolates from CF infections. Taken together, these results suggest that virulence and in particular pathogenicity-related genes in different organisms are both multifactorial and combinatorial, and that the outcome of a specific host-pathogen interaction depends on the bacterial origin as well as on the host genetic background. Recent whole genome sequence analyses of *P. aeruginosa* strains isolated from CF patients described loss-of-function mutations in virulence genes, suggesting attenuation of virulence for CF-adapted strains [13]. In the case of CF infections, *P. aeruginosa* clonal populations remain isolated in a defined environment over a long period of time and normally do not spread to other patients. Whether *P. aeruginosa* phenotypes that have adapted to the CF-niches, have lost their competitive fitness in other environment is not known.

To expand our knowledge on *P. aeruginosa* virulence and how this bacterium interacts with its host, we tested the hypothesis that CF-niche adaptation and specialization reduces the bacterial pathogenic potential of the organism in acute infection models. We selected well characterized *P. aeruginosa* clonal lineages of strains isolated from three CF patients at the onset of infections and after several years of chronic colonization; the samples included late adapted strains carrying several phenotypic changes in virulence factor production, structural modification in the Pathogen-Associated Molecular Patterns (PAMPs) [14,5], and patho-adaptive mutations within the genome temporally associated with CF lung infection [15]. These *P. aeruginosa* clonal lineages were tested in a multiple infection hosts, including *Caenorhabditis elegans*, *Galleria mellonella*, *Drosophila melanogaster* and mice with two different genetic backgrounds, C57bl/6NCrl and Balb/cAnNCrl, described previously as susceptible and resistant [16]. We showed that *P. aeruginosa* early strains were lethal in the multi-host models included in this study while late strains reduced or abolished acute virulence. Our findings suggest that the adaptation of different *P. aeruginosa* lineages within CF lungs selects populations with reduced pathogenic potential in acute infections which is maintained across a broad range of hosts.

## Results

### Pathogenic potential of *P. aeruginosa* sequential strains from CF patients in *C. elegans*, *D. melanogaster* and *G. mellonella*

*P. aeruginosa* longitudinal strains isolated from three CF patients at the onset of infection (early) and after several years of chronic colonization (late) and carrying several phenotypic differences (**Figure 1 and Table S1**) were tested for their virulence potential in three non-mammalian hosts, namely *C. elegans*, *D. melanogaster* and *G. mellonella*. In these experiments early *P. aeruginosa* strains, AA2, KK1, KK2 and MF1, and late strains, AA43, AA44, KK71, KK72 and MF51 were administered to non-mammalian hosts and mortality was monitored. *P. aeruginosa* early strain AA2 was significantly more lethal than the clonal late isolates AA43 and AA44 in *C. elegans* (AA2: 100% vs AA43: 21% and AA44: 41%, Mantel-Cox test: $p<0.001$) (**Fig. 2A**). Similar results were also obtained in *D. melanogaster* (AA2: 100% vs AA43: 82% and AA44: 97% $p<0.001$)(**Fig. 2D**). Although the late strains AA43 and AA44 were more pathogenic in this model in comparison to *C. elegans*, they killed the fruit flies later in comparison to the early strain AA2. Likewise, early *P. aeruginosa* isolates from patients KK and MF were significantly more lethal than their clonal late isolates in both models. However, lethality in *C. elegans* (KK1: 25% and KK2: 36% vs KK71: 11% and KK72: 7%, $p<0.01$; MF1: 69% vs MF51: 35%, $p<0.001$) (**Fig. 2B, C**) was generally less severe than in *D. melanogaster* (KK1: 100% and KK2: 100% vs KK71: 16% and KK72: 35%, $p<0.01$; MF1: 99% vs MF51: 11%, $p<0.001$) (**Fig. 2E, F**). We also evaluated lethality in a *G. mellonella* infection model. As in the previous models, the $LD_{50}$ of the early isolate AA2 was found to be more than 20-folds reduced when compared to the clonal late isolates AA43 and AA44 (**Table 1**). Similar trends were also seen with the sets of early and late isolates (KK and MF isolates). The $LD_{50}$ of the early isolates KK1 and KK2 were found to be more than 20-folds reduced when compared to the clonal late isolates KK71 and KK72. The early isolate MF1 showed a $LD_{50}$ of 1500-folds reduced when compared to the clonal late isolate MF51, confirming the higher acute virulence of early strains.

### Response of different C57Bl/6NCrl and Balb/cAnNCrl inbred mouse strains to infection with *P. aeruginosa* sequential strains

To test whether the differences in lethality between early and late clonal *P. aeruginosa* strains are maintained in the mammalian host, we analyzed the host response in murine models of acute pneumonia. Lethality and changes in body weight in C57Bl/6NCrl and Balb/cAnCrl inbred mouse strains were assessed. First,

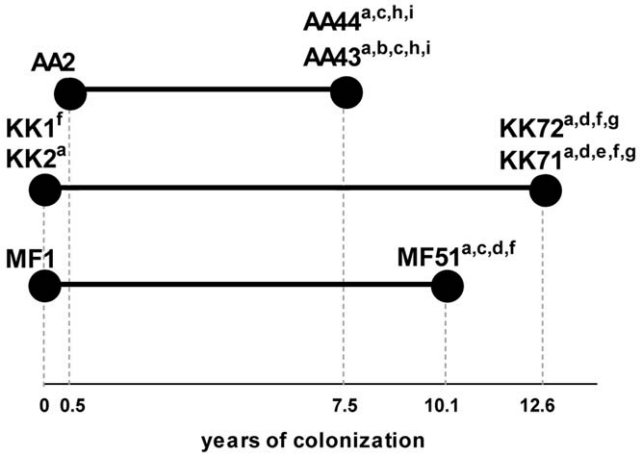


**Figure 1. Genotypic and phenotypic characteristic of *P. aeruginosa* sequential isolates from CF patients.** Three clonal lineages (AA, KK and MF) of *P. aeruginosa* strains were isolated at the onset of chronic colonization (early: AA2, KK1, KK2, MF1) or several years after acquisition and before patient's death (late: AA43, AA44, KK71, KK72, MF51). Clonality of strains was assessed by Pulsed Field Gel Electrophoresis and was reported previously [4]. Multiple phenotypic traits changed during genetic adaptation to the CF lung and included [14]: (a) motility defect, (b) mucoid phenotype, (c) protease reduction, (d) siderophore reduction, (e) hemolysis reduction, (f) LasR phenotype, (g) growth rate reduction. In addition, lipopolysaccharide (LPS) lipid A (h) and peptidoglycan (PGN) muropeptides (i) were analysed exclusively in the lineage AA showing specific structural modifications temporally associated with CF lung infection as described previously [5]. Additional data were reported in the online data supplement (**Table S1**).
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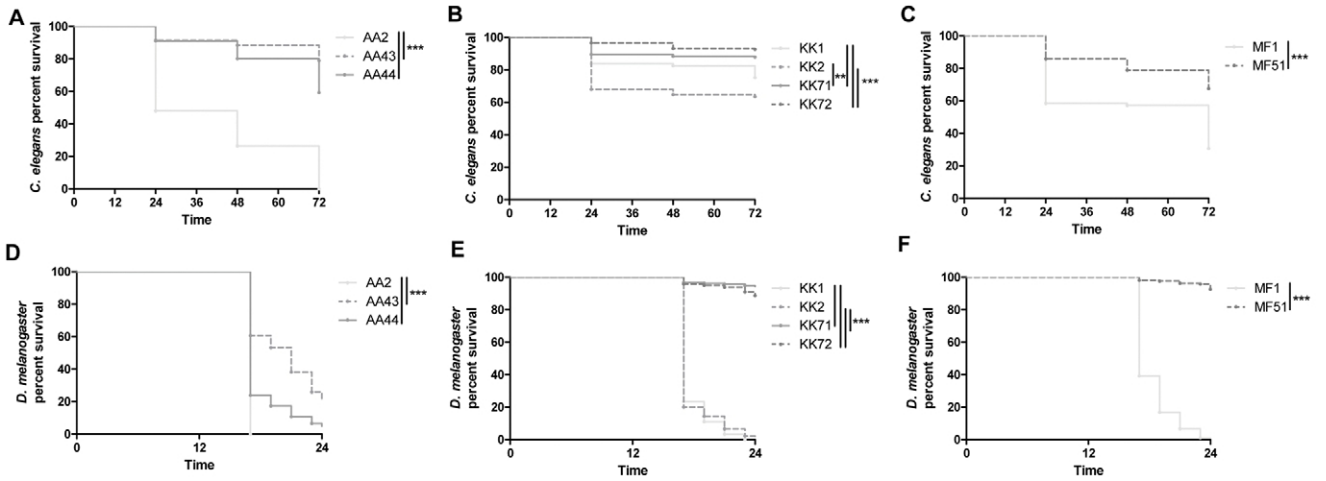


**Figure 2. Pathogenicity of clonal pair of early/late *P. aeruginosa* isolates in *C. elegans* and *D. melanogaster*.** Pathogenicity of lineages of early and late *P. aeruginosa* isolates in *C. elegans*: AA, (**A**); KK, (**B**); MF, (**C**); Pathogenicity of different lineages of early and late *P. aeruginosa* isolates in *D. melanogaster*: AA lineage, (**D**); KK lineage, (**E**); MF lineage, (**F**). Three independent experiments were pooled. Statistical analysis was calculated for pair wise comparisons between early and late strains (** $p<0.01$, *** $p<0.001$, Mantel-Cox test).
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escalating doses ranging from $10^5$ to $10^9$ cfu of *P. aeruginosa* were applied to C57Bl/6NCrl mice to determine the relative range of susceptibility. As shown in **Fig. 3** and **Table S2**, C57Bl/6NCrl died starting from $5\times10^6$ cfu/lung of early AA2 strain and $1\times10^7$ cfu/lung of early KK1 and KK2 strains, indicating differences in virulence between *P. aeruginosa* early strains of different lineages. When mice were inoculated at the same doses, late AA43, AA44, KK71 and KK72 strains were not lethal, indicating that their virulence was attenuated in comparison to the early strains (AA2 vs AA43 and AA44, $p<0.001$; KK1 and KK2 vs KK71 and KK72, Mantel-Cox, $p<0.001$) (**Fig. 4A and B**). In the AA lineage, all mice died at doses of $10^8$ cfu/lung of AA43 and AA44 strains (**Fig. 3A**), while in the KK lineage doses of $10^9$ cfu/lung of KK71 and $10^8$ cfu/lung of KK72 were fully lethal (**Fig. 3B**). Differences between early and late strains were also observed in BALB/cAnNCrl mice (AA2 vs AA43 and AA44, $p<0.05$; KK1 and KK2 vs KK71 and KK72, $p<0.01$) (**Fig. 4C and D**), which showed similar susceptibility as C57Bl/6NCrl. Bacterial cells were recovered from blood and other organs of moribund mice indicating that death was caused by sepsis (data not shown).

In accordance with these results, a major decrease in body weight was observed in mice infected with the early *P. aeruginosa* strains AA2, KK1 and KK2 when compared with the late clonal strains AA43, AA44 and KK71 both in C57Bl/6NCrl and Balb/cAnCrl (**Fig. S1**). Infections with KK72 strain appeared to be an exception.

**Table 1.** $LD_{50}$ of longitudinal clonal *P. aeruginosa* lineages in *G. mellonella* larvae 24 hours post infection.

| Strain | Nr. of Cells ($LD_{50}$) |
|---|---|
| AA2 | 15 |
| AA43 | $>3\times10^2$ |
| AA44 | $>3\times10^2$ |
| KK1 | $1.3\times10^3$ |
| KK2 | $2.8\times10^4$ |
| KK71 | $>6.0\times10^5$ |
| KK72 | $>7.0\times10^5$ |
| MF1 | $2.0\times10^2$ |
| MF51 | $>3.0\times10^5$ |

$>LD_{50}$ is higher than the maximum infection dosage used. Data represent mean values of at least three independent experiments.
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## Histopathological lesions, localization and quantification of *P. aeruginosa* strains in the murine airways

To assess clinical strain-specific traits of acute pneumonia, lung histopathology was performed on mice challenged with strains of the *P. aeruginosa* AA clonal lineage for 24 hours. This analysis revealed that acute infection with early AA2 strain caused more severe lesions and leukocytes recruitment in the airways than infection with late AA43 and AA44 (**Fig. 5 A–C, E–G**). The area infiltrated with inflammatory cells was significantly increased in the AA2 strain compared to AA43 and AA44 infected mice (cell infiltration mean±SEM: 63.99±5.42% of AA2 vs 43.15±0.91% of AA43 and 44.51±0.44% of AA44, Mann Whitney test, $p<0.05$) (**Fig. 5O**). Accordingly, the percentage of tissue preservation was significantly higher for AA43 and AA44 compared to AA2 (36.01±5.42% of AA2 vs 56.85±0.91% of AA43 and 55.49±0.44% of AA44, $p<0.05$).

Immunofluorescence staining showed that the early strain AA2 was localized both within the bronchial lumen and within alveolar space (**Fig. 5I**), supporting its spreading to other organs during sepsis. In contrast, the late strains AA43 and AA44 were localized exclusively within the bronchia (**Fig. 5L, M**). Next, we quantified the bacterial load in the lungs of mice up to 48 h post infection. Given a starting dose of $5\times10^6$, early AA2 strain replicated in the airways reaching a high load ($2.3\times10^8$ median CFU) and causing death of the animal (**Fig. 5P**). Late AA43 and AA44 decreased significantly bacterial numbers soon after infection and were completely cleared by the host immune system after 48 h, indicating a low pathogenic potential (AA2 vs AA43 and AA44, Student's t-test, $p<0.05$).

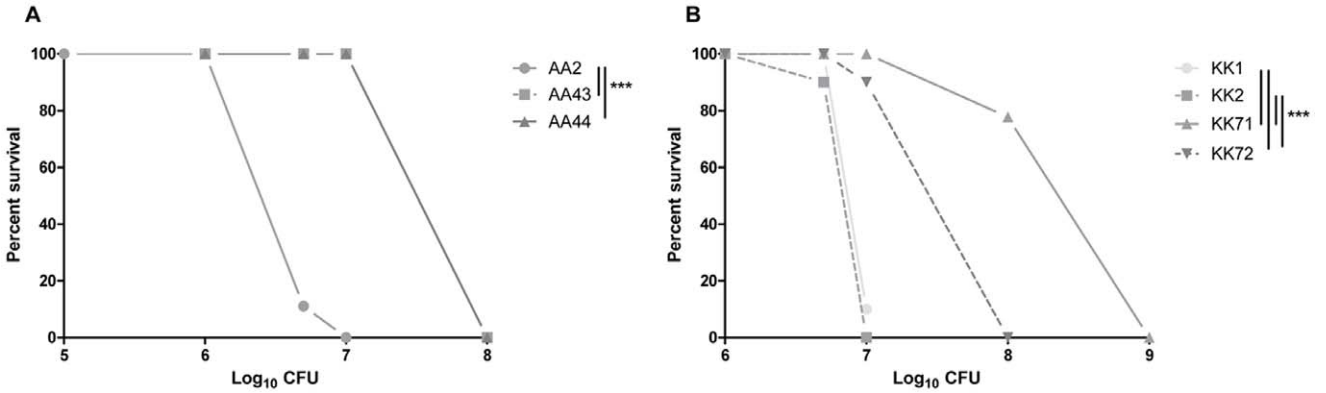


**Figure 3. Correlation between survival percent and initial infection dose of clonal pair of early/late *P. aeruginosa* isolates in C57Bl/6NCrl.** C57Bl/6NCrl mice were infected with different doses of *P. aeruginosa* strains from AA (**A**) and KK (**B**) clonal lineages. Survival of infected mice was followed over a period of 4 days and is indicated as a cumulative percent. Higher doses of late *P. aeruginosa* strains (AA43, AA44, KK71, KK72) are required for mortality when compared to early strains (AA2, KK1 and KK2). Two to three independent experiments were pooled (nr of mice: 5–18 as detailed in **table S2**). Statistical analysis of pair wise comparisons for early and late strains are indicated *** $p<0.001$ (Mantel-Cox test).
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## Invasion of *P. aeruginosa* sequential strains in epithelial cells

Bacterial invasion of host cells is a process common to many pathogens, including the CF-related pathogen, to evade extracellular immune factors [17] or to favour systemic spread [18]. We tested the ability of the *P. aeruginosa* clinical strains to invade CF respiratory cells (IB3-1) and isogenic corrected cells (C38). As shown in **Fig. 6**, early AA2 strain was found to be significantly more invasive than the *P. aeruginosa* clonal late strains AA43 and AA44 in both IB3-1 and C38 cells (IB3-1: AA2 vs AA43 $p<0.05$, AA2 vs AA44 $p<0.001$; C38: AA2 vs AA43 $p<0.001$, AA2 vs AA44, Student's t-test: $p<0.001$). In particular, AA44 strain was completely non-invasive in these experiments. Similar results were obtained with strains of the KK lineage. Early KK1 was significantly more invasive when compared to late KK71 and KK72 strains (IB3-1: KK1 vs KK71 and KK72, $p<0.01$ and $p<0.001$, respectively; C38: KK1 vs KK71 and KK72, $p<0.01$ and $p<0.001$, respectively). Early KK2 strain was more invasive than late clonal strains KK71 and KK72 both in IB3-1 and C38 cells, although significance was found only in IB3-1 cells (KK2 vs KK71, $p<0.01$).

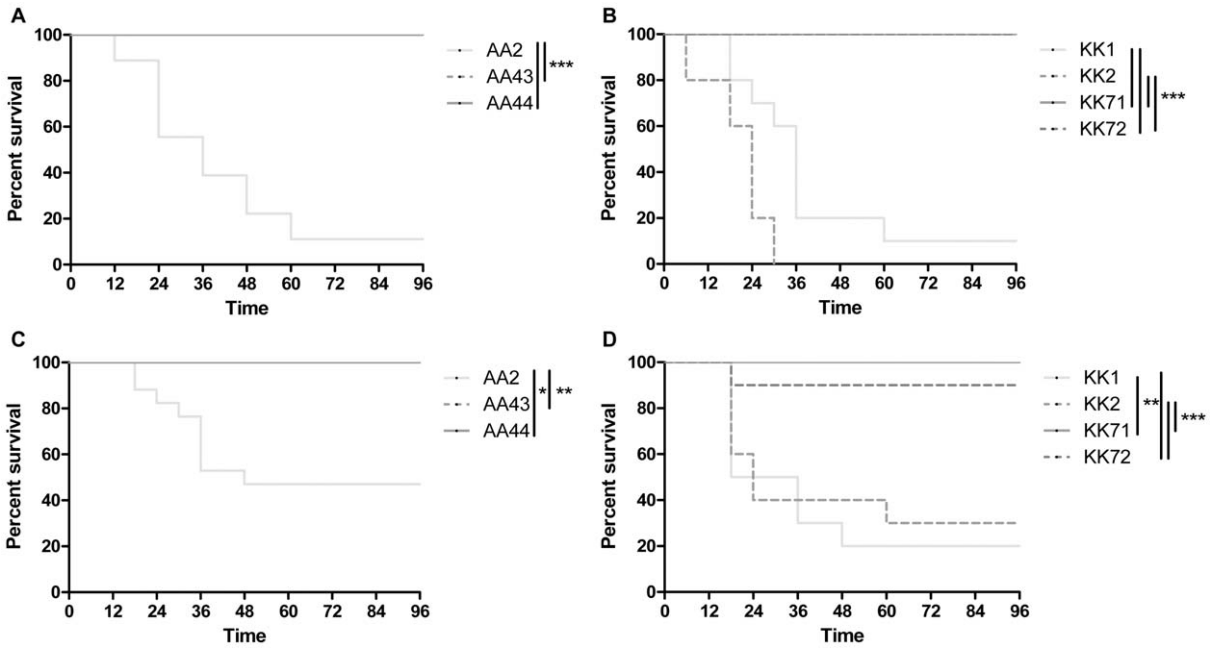


**Figure 4. C57Bl/6NCrl and BALB/cAnNCrl inbred mouse strains exhibit a similar susceptibility after infection with clonal pair of early/late *P. aeruginosa* isolates.** C57Bl/6NCrl (**A, B**) and BALB/cAnNCrl (**C, D**) mice were infected with $5\times10^6$ cfu/lung of *P. aeruginosa* strains from AA (**A, C**) and $1\times10^7$ cfu/lung KK (**B, D**) clonal lineages. Survival of infected mice was followed over a period of 4 days. Early strains (AA2, KK1 and KK2) were lethal while late strains (AA43, AA44, KK71, KK72) were attenuated in acute virulence. Two to three independent experiments were pooled (nr of mice: 5–18 as detailed in **table S3**). Statistical analysis was calculated for pair wise comparisons between early and late strains (* $p<0.05$; ** $p<0.01$; *** $p<0.001$, Mantel-Cox test).
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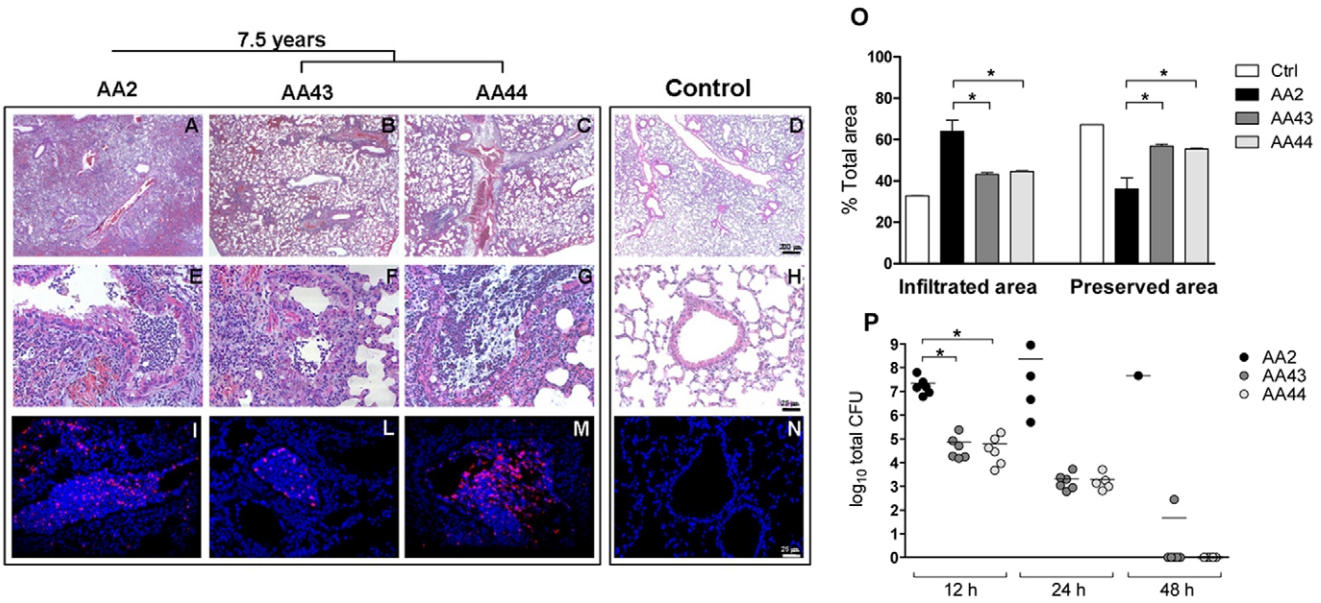


**Figure 5. Histopathology, *P. aeruginosa* load and localization in murine lungs after infection with clonal pair of early/late *P. aeruginosa* isolates.** C57Bl/6NCrl were infected with $5\times10^6$ cfu/lung of *P. aeruginosa* strains from AA lineage for 24 hours. Control mice were not infected. Lungs were stained with H&E and in immunofluorescence with specific antibody against *P. aeruginosa* (red) (**A, E, I**: AA2; **B, F, L**: AA43; **C, G, M**: AA44; **D, H, N**: not infected). Counterstaining was performed with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (blue). **I–N**) Bacterial cells of *P. aeruginosa* are visible in the bronchia and pulmonary parenchyma. O) Severity of lesions and lung involvement is heterogeneous in different lobes of the same mice. Quantification of infiltrated and preserved areas as percentage of total tissue area with mean ± SEM is shown (n = 3 mice each/strain). Statistical analysis was calculated for pair wise comparisons between early and late strains (* $p<0.05$; ** $p<0.01$; *** $p<0.001$, Mann–Whitney). **P**) Dots represent individual measurements of the no. of cfu per lung, and horizontal lines represent median values after 12, 24 and 48 h. Two independent experiments were pooled. Statistical analysis was calculated for pair wise comparisons between early and late strains (* $p<0.05$, Student's t-test).
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## Discussion

Previous studies that were based on whole genome sequence analyses of longitudinal *P. aeruginosa* isolate from CF patients suggested that bacterial invasive functions are selected against during the course of chronic infection [5,11]. Examples include motility, type III secretion system, O antigen biosynthesis, exotoxin, protease, and phenazine production, among others. Historically, many of these functions are considered to be virulence factors, as they provoke acute infection or dissemination within the host. Consequently, a major question that derived from previous reports and which requires further investigation was whether adaptation of *P. aeruginosa* strains to the CF-niche changes the fitness in other environments.

In this paper, we tested this hypothesis by evaluating acute pathogenicity of *P. aeruginosa* clonal variants from CF patients, in multiple infection hosts. The *P. aeruginosa* clonal strains included in this panel were isolated at different time points during CF chronic lung infection and were genetically characterized for genome

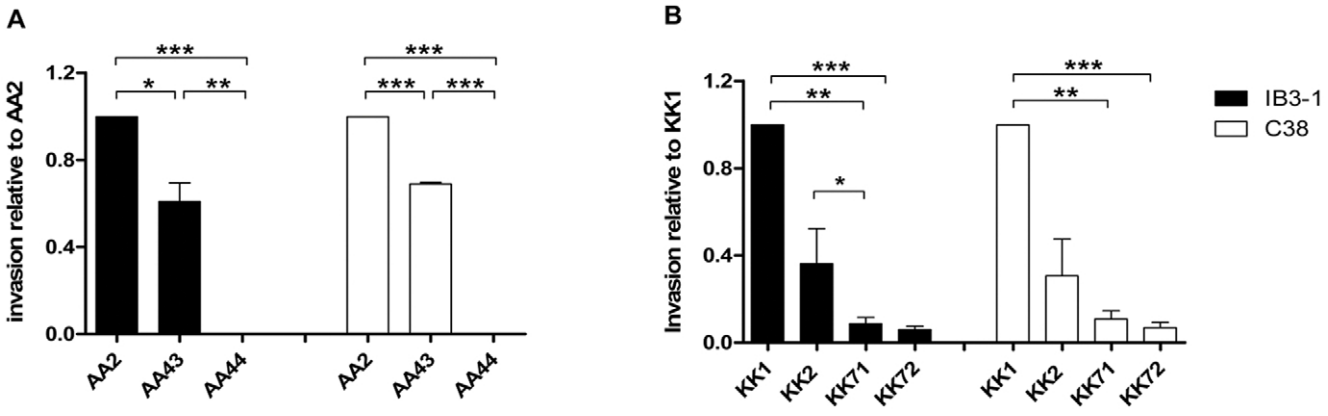


**Figure 6. Invasion of clonal pair of early/late *P. aeruginosa* isolates in IB3-1 and C38 cells. A**) Fold of invasion relative to AA2 after 1 h of stimulation with AA clonal lineage. **B**) Fold of invasion relative to KK1 after 1 h of stimulation with KK clonal lineage. Measurements were performed in triplicate. Statistical analysis was calculated for pair wise comparisons between early and late strains (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's t-test).
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rearrangements, mutations, and variations in pathogenic islands, and phenotypically for the loss of motility, acquisition of mucoidy, and a number of changes in the production of distinct virulence factors [14,15]. Furthermore, the *P. aeruginosa* late strains, which were selected for this study, clustered with respect to their ability to persist in CF airways as well as in murine models of chronic infection that mimic the anaerobic conditions found the CF sputum [19,14]. When *P. aeruginosa* longitudinal strains were tested in non-mammalian infection models, including *C. elegans*, *G. mellonella* and *D. melanogaster*, a reduction in acute virulence was observed in late strains relative to the respective early isolates. Most notably, there was a general agreement between the three models. When the panel of hosts was expanded to C57bl/6NCrl and Balb/cAnNCrl inbred mouse strains of different genetic backgrounds, we confirmed that early strains were lethal while late adapted *P. aeruginosa* strains were attenuated in acute virulence.

Based on previous reports on *P. aeruginosa* and other CF-related pathogens, this result was not obvious. In fact, the ability of bacteria to survive in a particular environment depends on virulence factors that are often specific for a particular host. Recent studies of *Burkholderia cenocepacia* infection in *C. elegans*, *G. mellonella*, alfalfa plant, mice and rats reported that most virulence factors are specific for one infection model only, and virulence factors are only rarely essential for full pathogenicity in multiple hosts. Only three factors were found to be essential for full pathogenicity in several host. *Burkholderia cenocepacia* mutants defective in quorum sensing, siderophore production and LPS biosynthesis were found to be attenuated in at least three of the infection models [20]. In *P. aeruginosa* PA14 strain only few host-specific virulence factors could be identified, and many of the mutants were attenuated in virulence in different hosts including *C. elegans*, *G. melonella* and mice [21]. However, when the virulence factors discovered in reference strains PA14 and PAO1 were tested in other clinical strains, no correlation between the absence and presence of these genes with virulence was observed [21]. Comparison of various clinical *P. aeruginosa* strains revealed that virulence is both multifactorial and combinatorial, the result of a pool of pathogenicity-related genes that interact in various combinations in different genetic background. *P. aeruginosa* clinical strains from the same type of infection exhibited a wide range of virulence in *C. elegans* [2]. For example, both the most and the least virulent strains tested were isolates from CF infections. Examination of specific genes among the several *P. aeruginosa* isolates did not reveal a consistent clustering of their genomic content with their pathogenic potential.

However, distinction between early and late *P. aeruginosa* strains from patients with CF had not been taken into account in previous works. Here, we directly compared *P. aeruginosa* early and late strains adapted to the CF-niche, which included strain exhibiting diverse phenotypes and belong to different genotypes, in several hosts. Although the genomes of *P. aeruginosa* isolates used in this work were not fully sequenced and only few phenotypic differences were identified, it is likely that late strains have accumulated several mutations during chronic persistence which account for the reduced pathogenicity across a broad range of hosts [14,15]. Thus, the genetic adaptation process that leads to CF-niche specialization restricts the overall virulence of late strains to other environmental niche. Regarding the *P. aeruginosa* strains selected in this study, this process is not strain-dependent but is consistent for all the late isolates.

We were unable to correlate the observed differences in virulence of early and late *P. aeruginosa* strains with a specific phenotype, but it is most likely that multiple mutations are responsible for the attenuation of late strain in acute virulence. Notably, a single phenotypic difference of the early KK1 and KK2 strains, including a LasR phenotype and a motility defect, did not change virulence in several hosts; major pathogenic differences are evident in KK71 and KK72, in which multiple phenotypic changes were observed. The mucoid AA43 and the non-mucoid AA44 strains did not differ in their virulence potential. In addition, AA43 and AA44 were similarly attenuated despite their differences in LPS lipid A, as has been reported previously [5].

Although rodents are the first choice for understanding infectious diseases in human, non-mammalian models can be useful surrogate hosts. *Drosophila* response to pathogens and mammalian innate immune defenses are characterized by pathways conserved in vertebrates [22]. *C. elegans* has been largely used to identify virulence factors [23], allowing the study of responses to infection as well as comparison of the virulence of clinical and environmental isolates [24]. More recently both model organisms, *C. elegans* and *Drosophila*, have been also used to study host tolerance in addition to resistance mechanisms [25]. Their innate immune system employs evolutionary conserved signalling pathways [26,21]. In reference to *G. mellonella*, it has been shown to correlate with mice models, when used to test *P. aeruginosa* virulence [27]. The use of non-mammalian infection models has several downsides, such as the specific temperature for the cultivation of the nematode may inhibit expression of certain virulence factors, the absence of the target organ and the lack of specific receptors or pathways. However, our results demonstrate the usefulness of these models for evaluating differences in acute virulence of *P. aeruginosa*.

Regarding the mammalian host, several studies have demonstrated that the host resistant/susceptibility response relies not only on the animal species but also on its genetic background [28,29,30,16]. In particular, different susceptibility to *P. aeruginosa* chronic bronchopulmonary infection has been reported among genetically well-defined inbred mouse strains when mice were exposed to clinical strains embedded in the agar beads. Based on the bacterial load detected in the lung after three days and two weeks, Balb/cAnCrl mice were found to be resistant and C57Bl/6NCrl mice were identified as susceptible in two different studies [31,28]. So far, direct comparison of the susceptibility of murine inbred strains to *P. aeruginosa* early and late strains from CF patients has not been performed. In our study, C57Bl/6NCrl and Balb/cAnCrl showed similar susceptibility to *P. aeruginosa* acute infection in terms of mortality but differences in pathogenicity among clonal early and late *P. aeruginosa* isolates observed in non-mammalian hosts. The genetic diversity of the mice in addition to the differences among type of infection (e.g. acute, reported in this work, and chronic, reported in previous works) and challenge, and bacterial origin may account for the different results [28,29,30,16,32]. Separate breeding colonies of C57Bl/6 mice maintained at the Charles River ("NCrl"), used in this study, or Jackson (J), used in previous studies, have led to the emergence of distinct substrains of C57Bl/6 mice that may explain the different susceptibility.

However, the findings that *P. aeruginosa* early strains were more lethal when compared to late strains in two different mouse genetic backgrounds strongly support the results in non-mammalian hosts that CF-niche adaptation of *P. aeruginosa* selects populations with reduced pathogenic potential in the acute infections. In addition, it has been argued that a high burden of infection but low virulence should account for host tolerance [33,34,25]. Consequently, our results indicated an increased host tolerance against *P. aeruginosa* CF adapted strains, as suggested by the high bacterial load sustained by the host. Our previous study showed that PAMPs of these *P. aeruginosa* strains, which were isolated at the late stage of

CF chronic infection, drastically impair the host immune detection system suggesting a role of adaptation in increasing host tolerance [5,35,25]. Histopathological analysis carried out in this work supports the previous findings that detection of *P. aeruginosa* adaptive strains is impaired compared to early strains. The mechanism(s) that permits *P. aeruginosa* to cause invasive infections with bacteremia or tolerance is not known. Some bacterial pathogens can induce their own uptake into host cells (invasion), allowing the pathogen to enter a protected niche and, in some cases, to pass through cellular barriers including the respiratory epithelium and/or the blood barrier [17,18]. Although further studies are needed to determine the exact mechanisms of *P. aeruginosa*/host interaction, it is tempting to speculate that the invasiveness of *P. aeruginosa* early strains may facilitate spreading from the lung to other tissues, while *P. aeruginosa* late strains, which are not able to protect themselves, may be finally eliminated.

Taken together, our results demonstrate that *P. aeruginosa* adaptation in CF airways selects pathoadaptive variants with a strongly reduced ability to cause acute infection processes in a host-independent way. These results have important implications for our understanding of the pathogenesis of *P. aeruginosa*-host interaction.

## Materials and Methods

### Ethics Statement

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

Research on the bacterial isolates from the individuals with CF has been approved by the responsible physician at the CF center at Hannover Medical School, Germany. All patients gave informed consent before the sample collection. Approval for storing of biological materials was obtained by the Hannover Medical School, Germany.

### Bacterial strains and CF patient

Nine sequential *P. aeruginosa* isolates from three CF patient carrying ΔF508/ΔF508 or ΔF508/R553X *cftr* mutation were chosen from the strains collection of the CF clinic Medizinische Hochschule of Hannover, Germany. Genotypic and phenotypic data of *P. aeruginosa* strains were published previously and summarized in **Figure 1** and **Table S1** [4,14,5]. *P. aeruginosa* was cultured in *Pseudomonas* isolation agar (PIA) or Trypticase Soy Broth (TSB) at 37°C.

### Investigation of pathogenicity in the *C. elegans* model

For the investigation of pathogenicity *C. elegans* strain DH26 has been used. Worms were synchronized into L4 larval stage by egg preparation, which was followed by incubation of isolated eggs on *E. coli* OP50 feeding plates at 20°C for around 76 hours. Subsequently, L4 larvae were transferred on the lawns of examined bacterial strains grown in the 6-well plates (approximately 30 worms per well) and incubated at 25°C. The surviving worms were counted after 24, 48 and 72 hours with the aid of a Stemi SV 6 microscope (Zeiss, Goettingen). The pathogenicity of the investigated bacterial strains was determined from the survival rates of *C. elegans* in three independent replicates.

### *G. mellonella* killing assays

Infection of *G. mellonella* larvae was performed as described previously [27], with some modifications. Caterpillars in the final larval stage (Brumann, Zurich, Switzerland) were stored in wooden shavings at 15°C and used within 2 to 3 weeks. Bacterial overnight cultures grown in LB broth were diluted 1:100 in 30 ml fresh medium and grown to an OD600 of 0.4 to 0.7. Cultures were centrifuged and the cellswere resuspended in 10 mM MgSO4 (E. Merck, Dietikon, Switzerland). 10-µl aliquots of three dilutions were injected into *G. mellonella* via the hindmost proleg using a 1-ml syringe (BD Plastipak, Madrid, Spain) with a 27-gauge needle (Rose GmbH, Trier, Germany).. Six healthy, randomly chosen larvae were injected and incubated at 30°C in the dark. As a control larvae were injected with 10 µl $MgSO_4$. The number of dead larvae was scored 24 h after infection and the $LD_{50}$ dosage was determined. Data are mean values for at least three independent experiments.

### Fly pathogenicity assay

Fly pricking assays were performed essentially as described by Apidianianakis *et al* [36]. 1 ml of an overnight culture was pelleted by centrifugation (10 min by 5000 rpm) and re-suspended in 1 ml of 10 mM $MgSO_4$ solution. A Tungsten stainless steel needle, with approximate diameters of 0.01 mm at the tip and 0.2 mm across the main needle body, was dipped into the bacterial solution and pricked into the middle dorsolateral thorax of anesthetized flies. For each strain 15 flies were used. As a control, the flies were pricked with $MgSO_4$ buffer. The infected flies were kept in glass vials, which were incubated at 26°C. Survival of the flies was monitored over time.

### Mouse model of acute *P. aeruginosa* infection

C57Bl/6 mice (20–22 gr) were purchased by Charles River. Mice were housed in filtered cages under specific-pathogen conditions and permitted unlimited access to food and water. Prior to animal experiments, the clinical *P. aeruginosa* strains were grown for 3 h to reach exponential phase. Next, the bacteria were pelleted by centrifugation (2700 g, 15 min), washed twice with sterile PBS and the OD of the bacterial suspension was adjusted by spectrophotometry at 600 nm. The intended number of cfu was extrapolated from a standard growth curve. Appropriate dilutions with sterile PBS were made to prepare the inoculum of $2\times10^6$ up to $2\times10^{10}$ cfu/ml. Mice were anesthetized and the trachea directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe according to established procedures [14] [37]. A 50 µl inoculum of $1\times10^5$ up to $1\times10^9$ cfu were implanted via the cannula into the lung, with both lobes inoculated. After infection, mortality and body weight were monitored in one group of mice over one week. In another group of mice, the lungs were excised, used for histopathology, homogenized and plated onto TSB-agar plates for cfu counting.

### Histological examination and immunofluorescence

Mice were sacrificed by $CO_2$ administration after 12, 24, 48 h of infection, lungs were removed en bloc and fixed in 10% buffered formalin at 4°C for 24 h, and processed for paraffin embedding. Longitudinal sections of 5 µm from the proximal, medial and distal lung regions were obtained at regular intervals using a microtome. Sections were stained with H&E according to standard procedures. Areas of inflammatory cell infiltration and tissue preservation (normal histology) were quantified using Image J software (National Institutes of Health) and reported as a percentage of total area [38]. Localization of *P. aeruginosa* was performed in de-paraffinized lung sections by employing a rabbit antiserum specific for *P. aeruginosa* and Texas Red-labeled goat anti-rabbit IgG as described [14]. The slides were examined using

an Axioplan fluorescence microscope (Zeiss), and images were taken with a KS 300 imaging system (Kontron).

### Cell cultures and invasion assay

IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (ΔF508/W1282X) and C38 cells, the rescued cell line which expresses a plasmid encoding a copy of functional CFTR, were obtained from LGC Promochem [39]. Cells were grown in LHC-8 media (Biosource) supplemented with 5% fetal bovine serum (FBS) (Cambrex Bio Science). All culture flasks and plates were coated with a solution of LHC-basal medium (Biosource) containing 35 μg/mL bovine collagen (BD Biosciences), 1 μg/mL bovine serum albumin (BSA, Sigma) and 10 μg/mL human fibronectin (BD Bio Science) as described [40].

Bacteria invasion assay was performed using Polymyxins B (100 μg/ml) (Sigma) protection assay with minor modifications [41]. *P. aeruginosa* strains, grown to the mid-exponential phase, were used to infect cell monolayers at a 100:1 multiplicity of infection for 1 h. The monolayers were washed with PBS, treated with antibiotic for 1 h, washed, lysed with $H_2O$ and plated on TSB-agar plates (Difco).

### Statistical analysis

Results are presented as median or mean±SEM. Student's t-test, Mann-Whitney test, Mantel-Cox test were used to determine the significance of differences in means between pairs.

## Supporting Information

**Figure S1 Weight change after infection with clonal pair of early/late *P. aeruginosa* isolates in C57Bl/6NCrl and BALB/cAnNCrl inbred mouse strains.** (**A**) C57Bl/6NCrl weights after infection with *P. aeruginosa* AA clonal lineage; (**B**) C57Bl/6NCrl weights after infection with *P. aeruginosa* KK clonal lineage; (**C**) BALB/cAnNCrl weights after infection with *P. aeruginosa* AA clonal lineage; (**D**) BALB/cAnNCrl weights after infection with *P. aeruginosa* KK clonal lineage. Data are expressed as mean ± SEM. Two to three independent experiments were pooled (nr of mice: 5–18 as detailed in table S3).
(TIF)

**Table S1 Genotypic and phenotypic characteristics of *P. aeruginosa* strains used in this work.**
(DOC)

**Table S2 Dose response in C57Bl/6NCrl infected with *P. aeruginosa* clonal lineages.**
(DOC)

**Table S3 Comparison between C57Bl/6NCrl and BALB/cAnCrl infected with *P. aeruginosa* clonal lineages.**
(DOC)

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## Author Contributions

Conceived and designed the experiments: NIL CC LE AB. Performed the experiments: NIL CC IDF CR MJ SS. Analyzed the data: NIL CC MJ SS. Contributed reagents/materials/analysis tools: LE AB. Wrote the paper: NIL CC LE AB.

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# Appendix 6

**The genetic basis of cadmium resistance of *Burkholderia cenocepacia***

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# The genetic basis of cadmium resistance of *Burkholderia cenocepacia*

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## Summary

***Burkholderia* species are highly resistant to heavy metals (HMs), yet their resistance mechanisms are largely unknown. In this study we screened 5000 mini-Tn*5* transposon insertion mutants of *Burkholderia cenocepacia* H111 for loss of cadmium tolerance. Of the four genes identified three affected outer membrane biogenesis and integrity or DNA repair. The fourth gene, BCAE0587, encoded a P1-type ATPase belonging to the CadA family of HM exporters. CadA-deficient strains lost the ability to grow in the presence of cadmium, zinc and lead, whereas resistance to nickel, copper and cobalt was not affected. Expression studies using a transcriptional fusion of the *cadA* promoter to *gfp* confirmed this specificity, as induction was only observed in presence of cadmium, zinc and lead. The promoter activity was found to be highest at neutral pH with an activation threshold of 30 nM cadmium. Inoculation of the HM-hyperaccumulating plant *Arabidopsis halleri* with a RFP-marked derivative of *B. cenocepacia* H111 containing the $P_{cadA}$–*gfp* fusion demonstrated the applicability of this biosensor for monitoring cadmium at the single cell level in a natural environment.**

## Introduction

Heavy metal (HM) contamination of soils is a worldwide environmental concern. Soil HMs are toxic for plants, animals and humans, as well as for microbes (Baath, 1989; Giller *et al*., 2009). Numerous studies have shown that the addition of HMs to soil causes a reduction of the microbial diversity and a change in the structure of the resident bacterial communities, with some genera becoming dominant over others (reviewed in Kozdroj and van Elsas, 2001). For example, it has been previously demonstrated that treatment of forest soil with cadmium strongly enriched the natural microbial community for *Burkholderia* species (Lazzaro *et al*., 2006; 2008; Schonmann *et al*., 2008).

Cadmium has been reported to be more toxic to microbes than copper, zinc and lead (Baath, 1989). Cadmium interferes with cysteine biosynthesis and binds to glutathione and the sulfhydryl groups of proteins, thereby inhibiting their function (Nies, 1999; Helbig *et al*., 2008). It has also been reported that cadmium causes DNA damage (Mitra and Bernstein, 1978; Badisa *et al*., 2007). Cadmium resistance mechanisms are diverse in bacteria and include metal exclusion via reduced membrane permeability, intra- or extracellular sequestration or active export (Bruins *et al*., 2000). Among these different possibilities active export has been suggested as the main mechanism in cadmium-tolerant bacteria. Four active export systems have been reported to confer resistance to cadmium in bacteria: CadA (Smith and Novick, 1972; Nucifora *et al*., 1989), Ncc (also exporting cobalt and nickel) (Schmidt and Schlegel, 1994), Czc (also exporting cobalt and zinc) (Nies, 1995), and the more recently discovered CzcP (Scherer and Nies, 2009). CzcP and Ncc were for a long time considered to be the major HM export systems in Gram-negative bacteria, while the P1-type ATPase CadA, which was first identified in *Staphylococcus aureus*, *Listeria* and *Lactococcus* species (Lebrun *et al*., 1994; Liu *et al*., 1997; Bal *et al*., 2003), was referred to as the 'efflux system of Gram-positive bacteria' (Silver and Phung, 1996). Only recently were export systems homologous to CadA identified in Gram-negative bacteria, e.g. in *Stenotrophomonas maltophilia*, *Pseudomonas putida* or *Escherichia coli* (Nies, 2003).

Although previous studies have shown that cadmium specifically selects for *Burkholderia* species, there is nothing known about the underlying molecular mechanism of cadmium resistance in this genus. The aim of the present study was therefore to identify the molecular determinant(s) of cadmium resistance in the model strain *Burkholderia cenocepacia* H111 (Romling *et al*., 1994). A screen of 5000 mini-Tn*5* transposon insertion mutants

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identified four genes that affected resistance to 1 mM $CdSO_4$: three of the genes encoded functions required for outer membrane biogenesis or DNA repair and thus influence the general fitness of the cell, while the fourth gene, which affected cadmium resistance most dramatically, was found to encode a CadA-family P1-type ATPase. We show that this exporter not only confers resistance to cadmium but also to high concentrations of zinc and lead and that expression of *cadA* is induced by the same three HMs. The *cadA* promoter region was used to construct a green fluorescent protein (GFP)-based cadmium biosensor, which was found to be induced when it colonized the roots of the HM-hyperaccumulating plant *Arabidopsis halleri*.

## Results and discussion

### *Identification of genes in* B. cenocepacia *required for cadmium resistance*

In order to identify the genetic determinants of cadmium resistance in *B. cenocepacia* H111, approximately 5000 random mini-Tn*5* transposon insertion mutants (Huber *et al*., 2002) were screened for lack of growth in LB medium supplemented with 1 mM $CdSO_4$. Seven of the mutants were unable to grow in medium supplemented with cadmium, while growth was unaffected in medium without $CdSO_4$. For each of these mutants, the DNA flanking the transposon insertion site was amplified by arbitrary PCR (O'Toole and Kolter, 1998), sequenced and blasted against the draft genome of *B. cenocepacia* H111 (European Nucleotide Archive NCAFQ00000000). In three mutants the transposon was found to be inserted at two different positions within the same gene (Fig. S1) and therefore our analysis identified four independent genes affecting cadmium resistance. The first gene, which was interrupted in mutants 35aA7 and 40G3, was identified as BCAE0587. This gene is predicted to encode a P-type ATPase of the CadA family, members of which have been demonstrated to export lead, cadmium, zinc and mercury and are known as major determinants of HM resistance in many, mostly Gram-positive, bacteria (Nies, 2003). In mutant 3G12 the transposon had inactivated BCAE0365, encoding a putative Δ9 fatty acid desaturase. Fatty acid desaturases catalyse the formation of a double bond in fatty acyl chains and are thus essential for proper membrane function in bacteria (Shanklin and Cahoon, 1998), suggesting that the higher cadmium susceptibility of this transposon mutant was a consequence of altered membrane permeability or stability. In two mutants (35aA12 and 8D3) the transposon had inserted at different positions in BCAE1998, which encodes a hypothetical protein that was identified in *Ralstonia eutropha* H16 as a DNA repair protein involved in the RecF pathway (Morimatsu and Kowalczykowski, 2003). As cadmium has been reported to induce DNA breakage (Mitra and Bernstein, 1978; Badisa *et al*., 2007), it is likely that the higher cadmium sensitivity of the two mutants is due to a defect in DNA repair. In fact, the two mutants were also found to be much more sensitive to UV radiation than the wild type (data not shown). The last gene identified in our screen was BCAE3201 (mutants 7G10 and 8E11), which encodes a protein that is homologous to TolR. The TolR protein is part of the Tol multiprotein complex, which plays a key role in the maintenance of outer membrane integrity and cell morphology in Gram-negative bacteria (Llamas *et al*., 2000). As with mutant 3G12, the cadmium sensitivity of these mutants is likely caused by a destabilized cell envelope.

In conclusion, three of the identified genes required for full cadmium resistance are essential for outer membrane biogenesis and integrity (BCAE0365, BCAE3201) or DNA repair (BCAE1998) and thus do not confer a specific resistance mechanism but rather affect cadmium uptake or the overall fitness of the cell. In this context it is important to note that although growth of these mutants was clearly impaired, it was not entirely abolished. This is in stark contrast to the two *cadA* mutants, which showed no growth at all in LB medium supplemented with 1 mM $CdSO_4$, indicating that this gene is the major player in cadmium resistance of *B. cenocepacia*. We therefore focused our subsequent investigations on *cadA*.

### *CadA confers resistance to cadmium, zinc and lead, but not to nickel, cobalt and copper*

We next constructed a defined *cadA* knock-out mutant, H111Δ*cadA*, as outlined in Appendix S1 and this strain was used along with the transposon insertion mutant to assess the role of *cadA* in HM resistance (Table S1). As a control we complemented mutant 35aA12 with a cosmid carrying the *cadA* wild type allele (Table S2). The various strains were tested for growth in a mineral base medium containing different concentrations of cadmium, zinc, lead, nickel, cobalt and copper. Growth of both *cadA* mutants was severely impaired in the presence of 2 mM cadmium, 2 mM lead and 5 mM zinc when compared with the wild type (Fig. 1). Expectedly, the growth defect was at least partially restored with the complemented mutant. In contrast, inactivation of *cadA* did not affect nickel, copper or cobalt resistance. While increasing cobalt concentrations led to decreased growth in all the strains tested, no growth inhibition was observed when the strains were grown with up to 5 mM nickel or copper (Fig. 1D; data not shown). The fact that mutants tolerated higher cadmium concentrations than in the original screening is likely to be due to the different

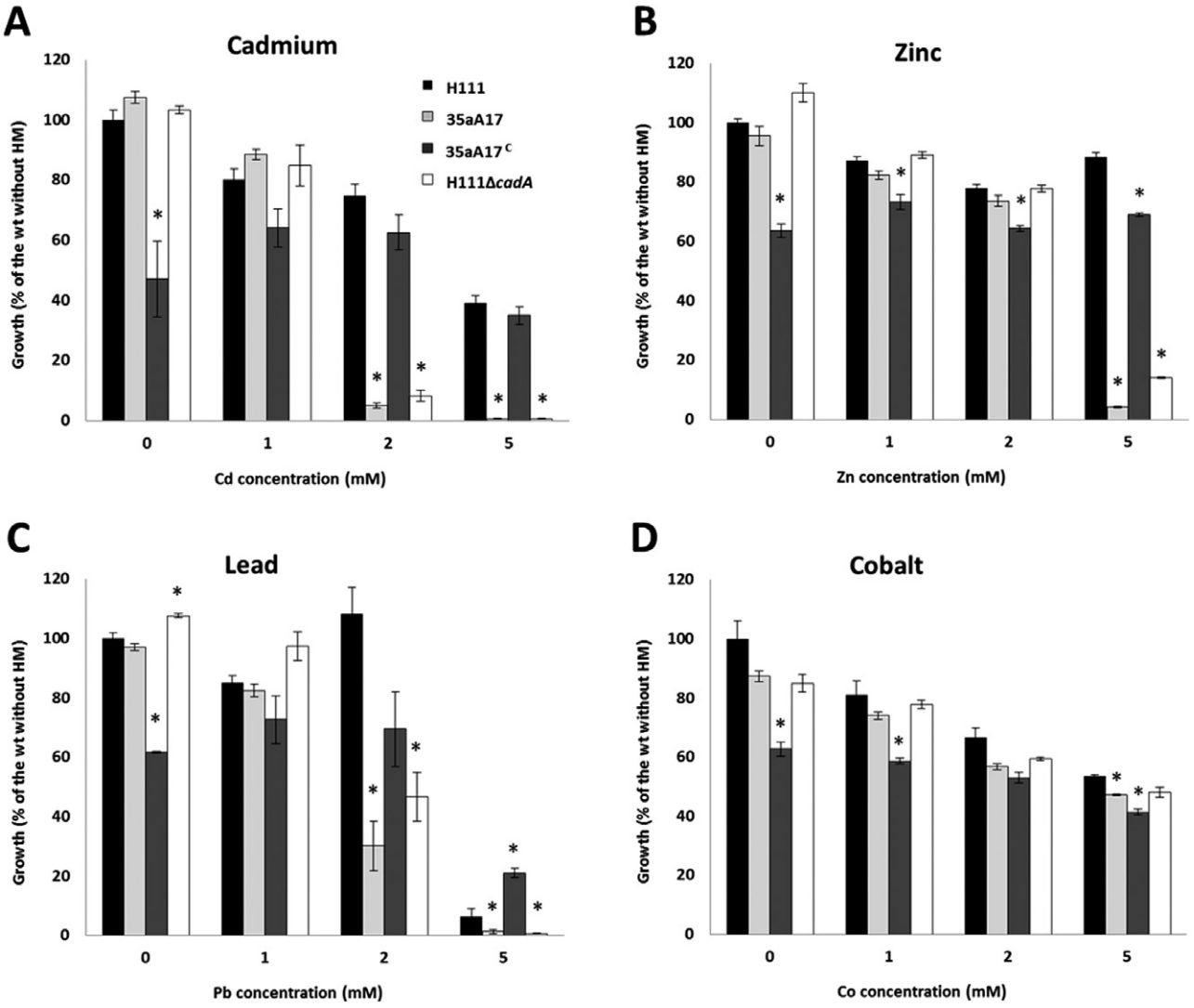


**Fig. 1.** Growth of the wild type *B. cenocepacia* H111 (black bars), the 35aA12 transposon mutant (35aA17, light grey), the complemented transposon mutant (35aA17$^C$, dark grey) and a defined *cadA* mutant (H111Δ*cadA*, white) was assessed in mineral base medium (adapted from Van Nostrand *et al*., 2005) containing different HMs in concentrations varying from 1 to 5 mM. Strains were grown overnight in mineral base medium at pH 7. The cultures were adjusted to an $OD_{600}$ of 0.005 in 96-well microtitre plates containing 200 μl of minimal base medium (pH 7) amended with different concentrations of $CdSO_4$ (A), $ZnSO_4$ (B), $Pb(NO_3)_2$ (C) or $CoCl_2$ (D). Plates were incubated overnight at 37°C and growth was measured by assessing the optical density at 600 nm. Results shown are percentages of the $OD_{600}$ obtained for the wild type in the absence of HM. Stars indicate statistical differences with the wild type (Student's *t*-test, $P < 0.001$, $n = 4$).

media used (mineral base medium versus LB) and/or to the biofilm lifestyle, which was favoured by the microtitre plate assay and might lead to a higher level of protection against HMs (Harrison *et al*., 2007).

### *Induction of* cadA *expression is HM-specific and pH-dependent*

To investigate expression of *cadA* we constructed a fusion of the *cadA* promoter region ($P_{cadA}$) to the GFP. As expected, expression was strongly induced in the presence of 1 mM cadmium, lead and zinc, but not by nickel, copper and cobalt (Fig. 2A). In agreement with previous reports (Van Nostrand *et al*., 2005; Worden *et al*., 2009), we noticed that HM resistance of *B. cenocepacia* H111 was higher when cells were grown at pH 5 than at pH 7 (data not shown), which is likely due to an increased competition of HM cations with protons at the cell surface (Franklin *et al*., 2000). To test whether pH also affected the induction of the $P_{cadA}$–*gfp* fusion we cultured the sensor strain at pH 5 and pH 7 in the presence of various $CdSO_4$ concentrations. These experiments revealed that at pH 7 the fusion was not only induced earlier but also at much higher levels than at acidic pH

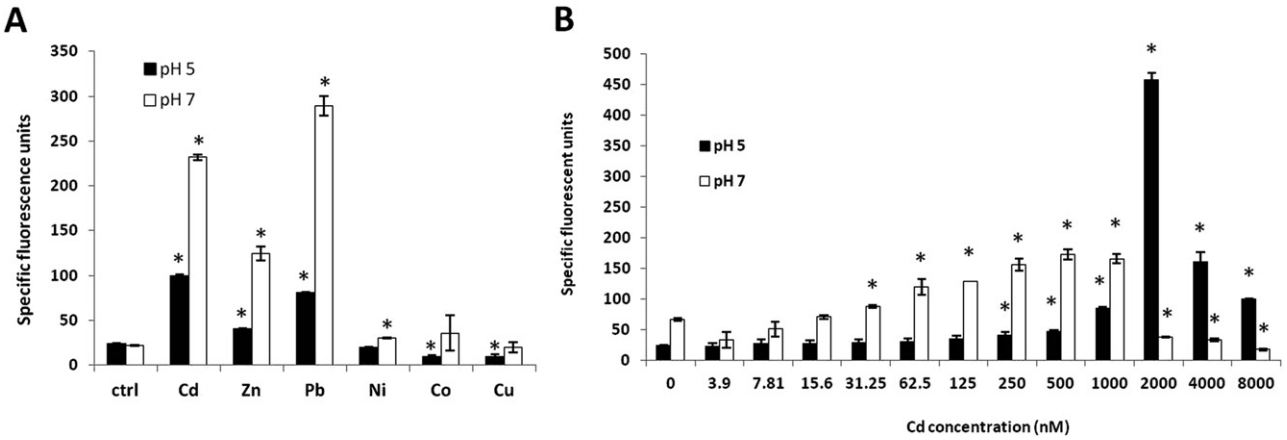


**Fig. 2.** Induction of *cadA* expression by different HMs. A plasmid carrying a transcriptional fusion of the *cadA* promoter with *gfp* was transferred into *B. cenocepacia* H111. An overnight culture of the strain was harvested, washed and inoculated into mineral base medium supplemented with 20 μg $ml^{-1}$ gentamicin (final $OD_{600}$ of 0.001) and 1 mM of either $CdSO_4$, $ZnSO_4$, $Pb(NO_3)_2$, $NiCl_2$, $CuSO_4$ or $CoSO_4$. Following 18 h incubation at 37°C the $OD_{600}$ was adjusted to 2.0 with 0.9% NaCl solution. Green fluorescence was measured at 528 nm in a spectrofluorometer microplate reader. Specific fluorescence units (fluorescence divided by $OD_{600}$) are shown. Expression of *cadA* in acidic (black bars) or neutral (white bars) mineral base medium containing 1 mM of either HM (A). $CdSO_4$ concentrations required for induction of *cadA* expression at acidic (black bars) or neutral (white bars) pH (B). Stars indicate statistically different values from the respective HM-free controls (Student's *t*-test, $P < 0.001$, $n = 2–3$).

(Fig. 2B), suggesting a quicker and higher stress response at neutral pH. These results demonstrate that expression of *cadA* is specifically triggered by cadmium, zinc and lead and that the expression level is dependent on pH.

### *Monitoring single cell expression of the* $P_{cadA}$–gfp *fusion on the roots of the HM-hyperaccumulating plant* A. halleri

Previous work has demonstrated that various organic and inorganic pollutants can be detected *in situ* by the aid of bacterial biosensors (van der Meer and Belkin, 2010). Although biosensors responding to various HMs with high sensitivity have been developed (Ivask *et al*., 2009), they have not been used for single cell expression studies, which may allow investigations of the spatial distribution of HMs in the sample. To test whether the $P_{cadA}$–*gfp* fusion is suitable for single cell analysis, we inoculated the reporter strain on the roots of *A. halleri* grown in hydroponic cultures in the absence or presence of 1 mM $CdSO_4$. In order to be able to localize the sensor strain on the root surface, it was chromosomally tagged with the red fluorescent protein (RFP). After 6 days, plant roots were inspected by confocal laser scanning microscopy. The sensor strain was found to form micro-colonies on the roots, independently of the presence or absence of cadmium (Fig. 3). Expectedly, only on the root surface of plants grown in medium supplemented with $CdSO_4$, the sensor cells also showed strong green fluorescence (Fig. 3). These data provide proof of principle that the reporter strain is well suited to *in situ* detect cadmium in a complex environmental sample at the resolution of a single bacterial cell.

## Conclusions

Although *Burkholderia* species are intrinsically resistant to HM and have been frequently retrieved from HM-contaminated environments (Kunito *et al*., 1997; Brim *et al*., 1999; Palmroth *et al*., 2007; Jiang *et al*., 2008; Lazzaro *et al*., 2008; Kuffner *et al*., 2010), knowledge on the underlying molecular mechanisms of HM resistance is scarce. Here we have shown that the P1-type ATPase CadA (BCAE0587) of *B. cenocepacia* H111 mediates resistance to cadmium, lead and zinc. CadA is highly conserved within the genus *Burkholderia*, including the plant endosymbiont *Burkholderia phytofirmans*, the plant pathogens *Burkholderia glumae* and *Burkholderia gladioli* and the human pathogen *Burkholderia pseudomallei*. Moreover, the *cadA* orthologues are in most species located close to the origin of replication of the main chromosome, a position that is typical for highly conserved core genes. As expected, the *cadA* gene is present in all sequenced *Burkholderia* genomes. Orthologues of *cadA* have also been described in various other bacteria, where they are often located on mobile elements, indicating that they are part of the accessory genome, i.e. only present in some adapted strains of a species. Although *Burkholderia* sp. is commonly found in soil, particularly associated with plant roots, and thus may frequently encounter high HM concentrations, it remains to

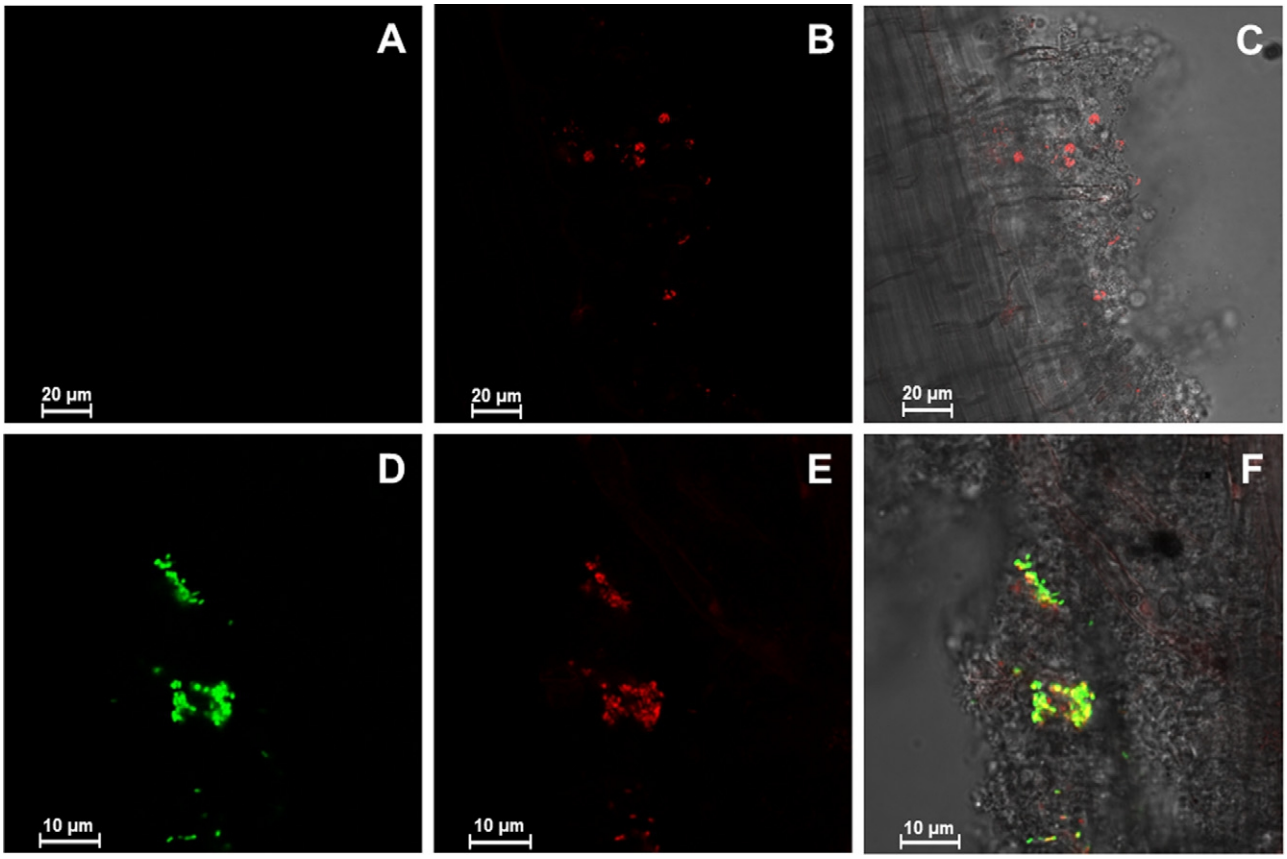


**Fig. 3.** Monitoring *cadA* expression on the roots of *A. halleri*. Seeds of *A. halleri* were surface sterilized, germinated and grown for 4 weeks on half-strength MS medium, after which they were transferred to hydroponic cultures. After 5 weeks the plants were exposed to 1 mM $CdCl_2$ and inoculated with RFP-tagged cells of *B. cenocepacia* H111 harbouring a plasmid carrying a *cadA::gpf* transcriptional fusion. Following 1 week of incubation the roots were inspected by confocal laser scanning microscopy (DM5500Q, Leica). Representative regions of roots from plants grown in either absence (A, B, C) or presence (D, E, F) of 1 mM $CdCl_2$ are shown. Green fluorescence (A, D) indicates *cadA* promoter activity; red fluorescence (B, E) shows the position of cells on the root surface. C and F show double exposure pictures that were overlaid with a phase contrast image.

be elucidated why CadA-mediated HM resistance is so highly conserved within the genus *Burkholderia*.

## Acknowledgements

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Identification of genes involved in cadmium resistance in *B. cenocepacia* H111. Screening of a Tn*5*-insertion mutant library resulted in seven mutants that had lost the ability to grow on LB medium supplemented with 2 mM $CdSO_4$. The DNA sequences flanking the transposon were determined using arbitrary PCR, essentially as described by O'Toole and Kolter (1998). Amplicons were sequenced and reads were mapped to the genome of *B. cenocepacia* H111.

**Table S1.** Strains used in this study.
**Table S2.** Plasmids used in this study.
**Appendix S1.** Experimental procedures.

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**Supplementary Material – Schwager et al.**

## Experimental Procedures

### Organisms and culture conditions

All bacterial strains used in this study are shown in Table S1. Bacteria were routinely grown in LB at 37°C with shaking. Antibiotics were added as required at the following concentrations: ampicillin (100µg/ml), kanamycin (50 µg/ml), chloramphenicol (25 µg/ml), and gentamycin (20 µg/ml). Growth of liquid cultures was monitored with an Ultraspec3100 Pro spectrophotometer (Biochron, Cambridge, England) by measurement of optical density at 600 nm. For plant experiments, seeds of *Arabidopsis halleri* were surface sterilized, germinated and grown on half-strength MS medium according to (Blom et al., 2011). After four weeks, they were transferred to hydroponic cultures (500 ml pots containing a solution containing the following nutrients (final concentration): 5mM $C_6H_{13}NO_4$ x $H_2O$, 3mM $CaNO_3$, 2mM $KNO_3$, 2mM Fe(III)EDTA, 0.8 mM $NH_4H_2PO_4$, 98 µM MgSO4 x $H_2O$, 4.6µM $H_3BO_3$, 0.5 µM $MnCl_3$ x $4H_2O$, 76nM $ZnSO_4$ x 7 $H_2O$, 32nM $CuSO_4$ x 5 $H_2O$, 11nM $MoO_3$. pH was adjusted to 6). The nutrient solution was aerated continuously and renewed every second week. Plants were grown in controlled conditions (16h/8h day/night and 20°C/15°C). Bacterial inoculation was performed as follows: 8 ml of an overnight culture of RFP-tagged *B. cenocepacia* H111 harbouring a *cadA::gfp* reporter plasmid were harvested, the pellet was resuspended in 2 ml 0.9% NaCl and inoculated into the plant pots.

### DNA manipulations, plasmid conjugation and sequence analysis of Tn*5* mutants

All plasmids used in this study are shown in Table S2. General molecular methods were as described by Sambrook (Sambrook, 1989). Plasmid DNA was routinely isolated using the

Qiagen miniprep kit. Following PCR amplification or restriction digestion, DNA was purified using the Qiagen PCR purification kit. Plasmids were introduced into *B. cenocepacia* H111 by conjugation. Bacterial conjugations were carried out using a filter mating technique (Herrero et al., 1990) . Briefly, donor, recipient, and helper E. coli HB101 (pRK600) or MM294 (pRK2013) were grown at 37°C overnight in 10 mL LB media supplied with the appropriate antibiotics. 2 mL overnight culture of each was harvested using a microcentrifuge (6000 rpm, 5 min), washed and resuspended in 500 µL LB. 100 µL donor cells and 100 µL helper cells were mixed and incubated at RT for 10 min. 200 µL recipient cells were added. The mixture was spot-inoculated onto the surface of prewarmed LB agar plates and incubated overnight at 37 °C. The cells were scraped off and resuspended in 1 mL 0.9% NaCl and plated on Pseudomonas Isolation Agar (DIFCO) medium containing antibiotics for counterselection of donor, helper and untransformed cells. For identification of the DNA sequences flanking transposon insertion sites in the transposon mutants, arbitrary PCR was carried out essentially as described by (O'Toole and Kolter, 1998). Briefly, a first round of PCR amplification was performed using the arbitrary primer ARB6 (5´-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3´) and the Tn5-transpon specific primer *luxC*ext2 (5´-AGTCATTCAATATTGGCAGG-3´). The first round PCR conditions consisted of (i) 5 min at 95 °C; (ii) 6 x [30 s at 95 °C, 30 s at 30 °C, 1 min at 72 °C]; (iii) 30 x [30 s at 95 °C, 30 s at 45 °C, 1 min at 72 °C]; (iv) 5 min at 72°C. The second round of PCR amplification used 5 µl purified first round PCR product as template and the following primers: ARB2 (5´-GGCCACGCGTCGACTAGTAC-3´) and *luxC*int2 (5´-GGATTGCACTAAATCATCAC-3´), with the following PCR conditions: (i) 30 x [30 s at 95 °C, 30 s at 45 °C, 1 min at 72 °C]; (ii) 5 min at 72 °C. Amplicons were sequenced using an ABI3730 automated sequencer (Applied Biosystems) and reads were mapped to the genome of *B. cenocepacia* H111 (WGS Accession No. CAFQ00000000).

**Construction of a cosmid library of *B. cenocepacia* H111 and complementation of the transposon mutant**

Chromosomal DNA of *B. cenocepacia* H111 was partially digested with *EcoR*I and ligated into the cosmid vector pLAFR3 cut with the same enzyme. In vitro packaging into *E. coli* HB101 was done by the aid of the Gigapack III Gold Packaging Reaction Kit from Invitrogen as described by the manufacturer. The library was transformed into mutant 35aA12 and transformants were selected on PIA medium containing 2 mM $CdSO_4$.

**Construction of a defined *cadA* mutant**

A 406 bp internal *cadA* fragment was amplified using the primer pair CadF417 (5'-GTTCCGGATCATGCAGATGG-3') and CadR825 (5'-GATGTTCAGGTTGCCGTTGC-3'). The amplicon was digested with NotI and cloned into the suicide vector pSHAFT2 cut with the same enzyme. The resulting plasmid was transferred to *B. cenocepacia* H111 by triparental mating and integrants were selected on PIA medium containing 90 µg/ml chloramphenicol. The correct genetic structure of the mutant was verified by PCR analysis.

**Measurement of *cadA* promoter activity**

A transcriptional fusion of the *cadA* promoter with *gfp* was constructed as follows: a 386 bp BamHI fragment of the BCAE0587 promoter region was PCR amplified using primers CadA_Pro_F (5'- AGGATCCGTGATCGTCGAGCAGC-3') and CadA_Pro_R (5'-TGGATCCGTATGACGGTGTCGTGG-3'). The amplicon was digested with BamHI (the introduced restrictions sites are underlined) and inserted into the promoter probe vector pGA-G1 cut with the same enzyme. The resulting plasmid, pMS1, was transferred to *B. cenocepacia* H111. An overnight culture of *B. cenocepacia* H111 (pMS1) was used to inoculate mineral base medium (adjusted to pH 5 or pH 7; $OD_{600}$ of 0.001; Van Nostrand et

al., 2005) supplemented with 1 mM citrate as carbon source, 20 µg/ml gentamycin and 1 mM of each of the following heavy metals: $ZnSO_4$, $CdSO_4$, $CoCl_2 \times 6H_2O$, $CuSO_4 \times 5\ H_2O$, $NiCl_2 \times 6\ H_2O$, $Pb(NO_3)_2$. Following 18 h of incubation at 37 °C, the $OD_{600}$ was measured and cultures were adjusted with 0.9 % NaCl to an $OD_{600}$ of 2.0. Green fluorescence was measured using a 300 µl sample in the microtitre plate reader SynergyTM HT (MWG Biotech, Germany) with an excitation wavelength of 485 nm and emission detection at 528 nm. The data were processed with KC4 software (BioTek Instruments). The fluorescence measurements were corrected for autofluorescence. Specific fluorescence units, i.e. relative fluorescence divided by the $OD_{600,}$ are shown.

86 Table S1. Strains used in this study.

87

| Species | | Description/ Characteristics | Source, Reference |
|---|---|---|---|
| ***Burkholderia cenocepacia*** | | | |
| | H111 | CF isolate | Romling et al. (1994) |
| | 35aA7 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 35aA12 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 40G3 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 3G12 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 7G10 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 8D3 | cadmium sensitive Tn*5* insertion mutant | This study |
| | 8E11 | cadmium sensitive Tn*5* insertion mutant | This study |
| | Δ*cadA* | BCAE0587 knock-out mutant, $Cm^R$ | This study |
| | dsRed | H111 tagged with pUT-Km-*dsRed*; $Km^R$ | Lumjiaktase et al., (2010) |
| ***Escherichia coli*** | | | |
| | HB101 | *recA thi pro leu hsdM*$^+$, $Sm^R$ | Boyer and Roulland (1969) |
| | DH5α | *sup*E44 *Δlac*U169 (Φ*lacZ*ΔM15) *hsd*R17 *end*A1 *rec*A1 *gyr*A96 *thi*1 *rel*A1 | Hanahan (1983) |
| | CC118 λpir | *Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 Δpir* lysogen | Herrero et al. (1990) |
| | MM294 | *hrs hrm*$^+$ *thi endA supE0* | Meselson and Yuan (1968) |
| | XL1 blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* | Bullock et al. (1987) |

88 Table S2. Plasmids used in this study.

| Plasmid | Characteristic | Source or reference |
|---|---|---|
| pGA-G1 | Broad host-range gfp-based promoter probe vector | Laboratory collection |
| pMS1 | pGA-G1 containing a $P_{cadA}$-gfp transcriptional fusion, $Gm^r$ | This study |
| pLAFR3 | Broad host-range cosmid vector, $Tc^R$ | Staskawicz et al. (1987) |
| pUT mini-Tn*5*Km2-*luxCDABE* | delivery vector for mini Tn*5*Km2-*luxCDABE,* $Km^R$ | Winson et al. (1998) |
| pGEM-T easy | multicopy cloning vector, $Ap^R$ | Promega |
| pRK600 | *ColE1oriV, RK-2Mob*$^+$ *RK2-Tra*$^+$, helper plasmid, $Cm^R$ | Kessler et al. (1992) |
| pRK2013 | ColE1 RK2-Mob$^+$ RK2Tra$^+$ derivative, $Km^R$ | Ditta et al. (1980) |
| pBBR1 MSC-5 | broad host-range cloning vector, $Gm^R$ | Kovach et al. (1995) |
| pSHAFT2 | suicide plasmid, *oriT*$^+$, *ori*R6K, $Cm^R$ | M. S. Thomas, unpublished |

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**Figure S1**

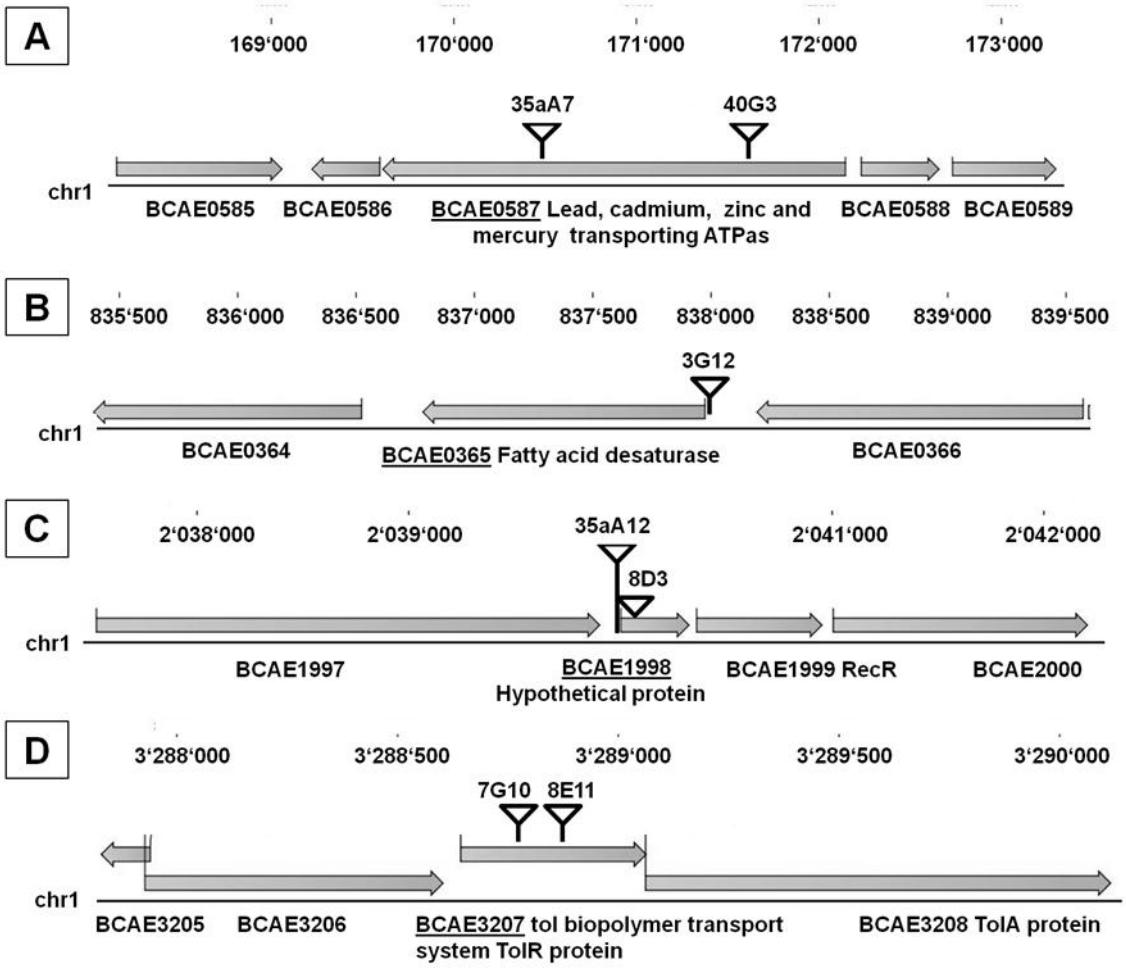


Figure S1. Identification of genes involved in cadmium resistance in *B. cenocepacia* H111. Screening of a Tn*5*-insertion mutant library resulted in seven mutants that had lost the ability to grow on LB medium supplemented with 2mM $CdSO_4$. The DNA sequences flanking the transposon were determined using arbitrary PCR, essentially as described by (O'Toole and Kolter, 1998). Amplicons were sequenced and reads were mapped to the genome of *B. cenocepacia* H111.

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# 9 Curriculum vitae

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| 2008 - 2012 | Doctoral work in the group of Prof. Dr. Leo Eberl at the Department of Microbiology, Institut of Plant Biology, Universitiy of Zürich, Switzerland |
| 2007 - 2008 | Master of Science in microbiology work in the group of Prof. Dr. Leo Eberl at the Department of Microbiology, Institut of Plant Biology, Universitiy of Zürich, Switzerland |
| 2003-2007 | Bachelor study in biology at the University of Zurich |
| 2001-2003 | Biochemistry studies at the ETH of Zurich |
| 1997-2001 | Matura Typus E at the Gymnasium in Immensee, Immensee |

Teaching Experience

| | |
|---|---|
| 2008-2011 | Teaching in laboratory Blockkurs BIO284 |
| 2008-2011 | Teaching undergrad courses BIO132 |
| 2010 | Supervising of a Master student working on her own project |
| 2009 | Supervision of a winter school student (six weeks) |
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## 9.1 Publications

### 9.1.1 Paper publications:

**Schwager, S., Agnoli, K., Köthe, M., Friederike, F., Givskov, M., Carlier, A. & Eberl, L. (2012).** Identification of *Burkholderia cenocepacia* H111 virulence factors using non-mammalian infection hosts. *Infection and Immunity, published online ahead of print on 22 October 2012*.

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### 9.1.2 Oral presentations:

2012 Stephan Schwager, Kirsty Agnoli and Leo Eberl. INVESTIGATION OF THE ROLE OF PC3 IN THE VIRULENCE OF BCC SPECIES, IBCWG 18th -21th April, Montreal, Canada

2011 Stephan Schwager, Anugraha Mathew, Aurelien Carlier, Pamela A. Sokol and Leo Eberl. IDENTIFICATION OF *BURKHOLDERIA CENOCEPACIA* H111 VIRULENCE FACTORS BY USING MULTIPLE INFECTION HOSTS. 4th MIM Retreat 4th – 6th September, Chandolin, Switzerland

2011 Stephan Schwager, Anugraha Mathew, Aurelien Carlier, Pamela A. Sokol and Leo Eberl. IDENTIFICATION OF BURKHOLDERIA CENOCEPACIA H111 VIRULENCE FACTORS BY USING MULTIPLE INFECTION HOSTS. IBCWG 13th – 16th April, Prague, Czech Republic

2011 Stephan Schwager and Leo Eberl. IDENTIFICATION OF POTENTIAL DRUG TARGETS IN *BURKHOLDERIA CENOCEPACIA* STRAINS BY USING MULTIPLE INFECTION HOSTS. NABATIVI (Novel approaches to bacterial target identification, validation and inhibition). 1th – 4th March, Brussels, Belgium

2010 Stephan Schwager, Leo Eberl. *DROSOPHILA MELANOGASTER:* A NEW INFECTION HOST FOR ASSESSING THE PATHOGENICITY OF *BURKHOLDERIA* SP. 3th MIM Retreat 18th - 20th of September, Ascona, Switzerland

### 9.1.3 Poster presentations:

2010 Stephan Schwager, Susanne Uehlinger and Leo Eberl. *DROSOPHILA MELANOGASTER:* A NEW INFECTION HOST FOR ASSESSING THE PATHOGENICITY OF *BURKHOLDERIA* 69th annual congress of the Swiss Society Microbiology (SSM-SGM), 24th – 25th June, Zürich, Switzerland

2009 Stephan Schwager, Susanne Uehlinger and Leo Eberl. FAST SCREENING OF THE PATHOGENICITY OF *BURKHOLDERIA* SPECIES BY USING NON-MAMMALIAN ANIMAL MODELS. 2th MIM Retreat 4th – 6th September, Parpan, Switzerland